THE UNIVERSITY
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The role of perinatal inflammation in preterm brain injury

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I. Abstract

Perinatal inflammation is associated with an increased risk of brain injury and neurodevelopmental impairment in preterm infants but the immune mediators driving this association are not well understood. This PhD thesis seeks to further characterise the inflammatory response associated with preterm birth, describe the relationship between perinatal inflammation and white matter development and explore the effect of specific inflammation-associated proteins on the development of human cortical neurons derived from induced pluripotent stem cells (iPSCs).

In the first study, I investigated the inflammatory profile at birth in 55 very preterm infants, compared to 59 term-born controls and then used this profile to predict exposure to intrauterine inflammation in the preterm group. Preterm infants had a distinct pro-inflammatory profile in umbilical cord blood at delivery when compared to term-born controls and IL-8 was found to be the strongest predictor of intrauterine inflammation.

In the second study, I investigated the association between specific inflammation-associated proteins and white matter microstructure in 71 very preterm infants using structural MRI and diffusion-weighted imaging of the brain. Elevated IL-8 in the first week of life was associated with white matter dysmaturation at term-equivalent age.

Following this discovery, I investigated the effect of IL-8 on the maturation and morphology of iPSC-derived cortical neurons and found that exposure was associated with impaired neurite outgrowth.

This thesis provides further evidence to support the role of inflammation in the aetiology of preterm brain injury and suggests that IL-8 dysregulation may link systemic inflammation with atypical cortical development and white matter disease in preterm infants.

II. Lay Summary

Preterm birth (before 37 weeks of gestation) affects approximately 11% of births globally each year. Some babies who are born too soon can encounter problems with learning, thinking and behaviour as they grow up due to altered brain development. We know that infection or inflammation around the time of birth can increase the risk of developmental problems but because our immune system responses to infection are complex, the specific mediators driving this association are not well understood.

This thesis presents three studies which characterise the inflammatory profile associated with preterm birth and the role of specific immune proteins on brain development.

In the first study I found that preterm infants have higher levels of pro-inflammatory proteins in their umbilical cord blood at delivery when compared to healthy term-born infants. I also found that a protein generated by the immune system in response to infection, known as interleukin-8 (IL-8), can predict which babies were exposed to infection before they were born.

In the second study I found that IL-8 measured from blood taken in the first week of life was associated with altered development of the major information highways of the brain in preterm infants who had a brain MRI performed around their due date.

In the final study I used human stem cells to generate neurons (specialised nerve cells which transmit and receive information from other nerve cells) and found that when they were exposed to IL-8 during early development, they did not grow as well.

These studies show that the immune system is involved in altered brain development in preterm infants and suggest that therapies to reduce inflammation may restore healthy brain development after preterm birth.

III. Dedication

To my creators Ed and Carol,

One who had remarkable foresight in the darkest of times and the other left alone with the responsibility of seeing it through. Frequently having to navigate uncharted territory but always with courage, love and devotion.

With gratitude beyond words, may your sacrifices benefit generations to come.

IV. Acknowledgements

At the end of my clinical training in Neonatology, embarking upon this PhD journey was a leap of faith that turned out to be one of the best decisions I have ever made. This is certainly just the beginning and there are many wonderful people to acknowledge for making this possible.

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Thank you to the many talented scientists, managers and students I have had the pleasure of working and collaborating with. Thanks to Jill for study organisation, David for keeping me fully stocked, Karen for stem cell expertise, James for assistance with cell profiling, Nis and Kristin for the customised immunoassay, Alan and Michael for imaging expertise and Margaret for placental reporting. The Radiographers at the Edinburgh Imaging Facility also deserve acknowledgement for outstanding teamwork and professionalism in the successful acquisition of images from such a challenging (but lovable) population.

I am grateful for the significant efforts of the recruitment team (if you know, you know). To my work wife and fellow baby whisperer, Gillian, and my favourite error prone perfectionist, Dave, thank you for your enthusiasm and commitment to the project. The many nights and weekends of co-ordinating consent, deliveries and sample collection were a small price to pay.

A special thanks to Manuel, Paola and Bhuvaneish for being so generous with your time and energy. I enjoyed our scientific discussions in the most unexpected of venues, from smashing crabs in Baltimore to sampling piña coladas in Puerto Rico (but mostly on the number 49 bus).

Thank you to my funders, Theirworld, for their generous investment and ongoing commitment to ensuring that every child has the best start in life. With that in mind, to the babies and families who took part in the study, I look forward to watching you grow and develop as we continue to learn together.

And finally, a special thanks to Antonio, a truly unexpected PhD discovery. We survived thesis writing together during a global pandemic. Here's to the next adventure.

V. Declaration

I declare that the content of this thesis is my own work and that all contributions and collaborations have been explicitly acknowledged in the text. No material presented here has been submitted for any other degree or professional qualification.

Gemma Sullivan
October 2020

VI. Publications arising from this thesis

1. **Sullivan, G.,** Galdi, P., Cabez, M.B., Borbye-Lorenzen, N., Stoye, D.Q., Lamb, G.J., Evans, M.J., Quigley, A.J., Thrippleton, M.J., Skogstrand, K., Chandran, S., Bastin, M.E., Boardman, J.P., 2020. Interleukin-8 dysregulation is implicated in brain dysmaturation following preterm birth. Brain, behavior, and immunity 90, 311-318.
2. Blesa, M., **Sullivan, G.,** Anblagan, D., Telford, E.J., Quigley, A.J., Sparrow, S.A., Serag, A., Semple, S.I., Bastin, M.E., Boardman, J.P., 2019. Early breast milk exposure modifies brain connectivity in preterm infants. NeuroImage 184, 431-439.
3. Blesa, M., Galdi, P., **Sullivan, G**., Wheater, E.N., Stoye, D.Q., Lamb, G.J., Quigley, A.J., Thrippleton, M.J., Bastin, M.E., Boardman, J.P., 2020. Peak Width of Skeletonized Water Diffusion MRI in the Neonatal Brain. Frontiers in neurology 11, 235.
4. Galdi, P., Blesa, M., Stoye, D.Q., **Sullivan, G**., Lamb, G.J., Quigley, A.J., Thrippleton, M.J., Bastin, M.E., Boardman, J.P., 2020. Neonatal morphometric similarity mapping for predicting brain age and characterizing neuroanatomic variation associated with preterm birth. NeuroImage Clinical 25, 102195.
5. Boardman, J.P., Ireland, G., **Sullivan, G.,** Pataky, R., Fleiss, B., Gressens, P., Miron, V., 2018. The Cerebrospinal Fluid Inflammatory Response to Preterm Birth. Frontiers in physiology 9, 1299.
6. Stoye, DQ., Blesa, M., **Sullivan, G.,** Galdi, P., Lamb, GJ., Black, G., Quigley, AJ., Thrippleton, M.J., Bastin, M.E., Reynolds, RM., Boardman, J.P. Maternal hair cortisol is associated with neonatal amygdala microstructure and connectivity in a sexually dimorphic manner. eLife. 2020; 9: e60729.
7. Blesa, M., Galdi, P., Cox S., **Sullivan, G**., Stoye, D.Q., Lamb, G.J., Quigley, A.J., Thrippleton, M.J., Escuardo J., Bastin M.E., Smith K.M., Boardman J.P. Hierarchical complexity of the macro-scale neonatal brain. Cerebral Cortex. 2020.

VII. Presentations arising from this thesis

1. IL-8 dysregulation is implicated in preterm brain injury.
   Paediatric Academic Societies Summer Webinar Series 31st July 2020

2. Structural connectivity measures correlate with language outcomes in preterm infants at 2 years of age.
   Neonatal Society Meeting, London 15th March 2019
   (Winner of best trainee oral presentation)
   Paediatric Academic Societies, Baltimore 28th April 2019

3. Early breastmilk exposure modifies brain connectivity in preterm infants.
   Neonatal Society Meeting, London 9th November 2017
   (Winner of best trainee oral presentation)
   Paediatric Academic Societies, Toronto 7th May 2018

VIII. Abbreviations

AD	Axial Diffusivity
ASD	Autism Spectrum Disorder
ADHD	Attention Deficit Hyperactivity Disorder
BSID-III	Bayley Scales of Infant Development, Third Edition
BPD	Bronchopulmonary dysplasia
CSF	Cerebrospinal Fluid
CP	Cerebral palsy
DAMP	Damage associated molecular pattern
DBSS	Dried Blood Spot Sample
DEHSI	Diffuse excessive high signal intensity
DTI	Diffusion Tensor Imaging
EOS	Early Onset Sepsis
FA	Fractional Anisotropy
FIRS	Fetal inflammatory response syndrome
FOV	Field of View
GA	Gestational Age
HARDI	High angular resolution diffusion imaging
HCA	Histologic Chorioamnionitis
IP	Intermediate progenitor
IQ	Intelligence quotient
IVH	Intraventricular Haemorrhage
LOD	Level of Detection
MD	Mean Diffusivity
MPRAGE	Magnetisation prepared Rapid Acquisition Gradient Echo
MRI	Magnetic Resonance Imaging
NDI	Neurite Density Index
NEC	Necrotising Enterocolitis
NODDI	Neurite Orientation Density and Dispersion Imaging
NPC	Neural progenitor cell
ODI	Orientation dispersion index
OL	Oligodendrocyte

OPC	Oligodendrocyte progenitor cell
PAMP	Pathogen associated molecular pattern
PMA	Post-menstrual age
PRR	Pattern recognition receptor
PSMD	Peak Width Skeletonised Mean Diffusivity
PSNDI	Peak Width Skeletonised Neurite Density Index
PVL	Periventricular leukomalacia
PWML	Punctate white matter lesions
RD	Radial Diffusivity
RDS	Respiratory distress syndrome
RG	Radial glia
ROI	Region of interest
ROP	Retinopathy of Prematurity
SVZ	Subventricular zone
TEA	Term-equivalent age
VZ	Ventricular zone

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1 Introduction

1.1 The global burden of preterm birth

1.1.1 Epidemiology

Preterm birth is defined as live birth before 37 completed weeks of pregnancy (WHO 1977). This can be classified as extremely preterm (<28 weeks), very preterm (28-32 weeks) and moderate to late preterm (32-37 weeks) (Blencowe et al. 2012). In 2014, approximately 14.84 million babies were delivered preterm worldwide, indicating a global preterm birth rate of 10.6% (Chawanpaiboon et al. 2019). Complications related to preterm birth are the leading cause of mortality in children under 5 years (Blencowe et al. 2013) and are responsible for 35% of deaths in the neonatal period (UN 2019). Preterm birth can be classified into deliveries that were indicated due to maternal, fetal or placental complications and those that followed spontaneous preterm labour. Many risk factors are known to be associated with preterm birth including maternal demographics, assisted reproductive technology, pregnancy complications, previous preterm birth and psychosocial influences but the cause is not always fully understood (Goldenberg et al. 2008; Barros et al. 2015; Ferrero et al. 2016).

1.1.2 Consequences of preterm birth

Whilst there have been significant advances in survival at the lowest gestational ages, there have not been significant improvements in the prevalence of major neonatal morbidities including bronchopulmonary dysplasia, necrotising enterocolitis, sepsis and brain injury (Costeloe et al. 2012; Fanaroff et al. 2007; Shah et al. 2012; Chen et al. 2016). Cohort studies have shown that amongst infants born very preterm there is a 19-35% incidence of moderate to severe disability in early childhood (Moore et al. 2012; Serenius et al. 2013; Pierrat et al. 2017; Rysavy et al. 2015; Draper

et al. 2019). This includes motor problems, cognitive impairment and sensory deficits (Johnson and Marlow 2017).

Whilst cerebral palsy (CP) affects 5-10% of survivors the rates of motor impairment without CP (balance, co-ordination, fine motor skills) remain significant, with very preterm infants scoring 0.57-0.88 SD below term-born peers in standardised tests of motor development in childhood (Spittle et al. 2018; Reid et al. 2016; Bolk et al. 2018; de Kieviet et al. 2009).

Preterm infants are at increased risk of neurocognitive impairment including lower IQ, executive dysfunction, and difficulties with working memory and language processing (Anderson 2014; Allotey et al. 2018; Brydges et al. 2018; Twilhaar et al. 2018; van Noort-van der Spek, Franken, and Weisglas-Kuperus 2012; Mulder et al. 2009). This has a pervasive effect on educational outcomes and academic performance (Twilhaar et al. 2018; Pettinger et al. 2020).

Preterm born children also exhibit a behavioural phenotype characterised by inattention, anxiety, peer problems and social withdrawal (Johnson and Marlow 2011; Ritchie, Bora, and Woodward 2015; Burnett et al. 2019). Neuropsychiatric disorders such as autistic spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) are more prevalent (Burnett et al. 2011; Agrawal et al. 2018; Franz et al. 2018; Montagna et al. 2020) and there is an increased risk of hospitalisation for psychosis, bipolar disorder or depression in adulthood (Nosarti et al. 2012).

Preterm birth has long-term consequences on adult functioning (Breeman et al. 2015). Cognitive deficits and attention problems persist into adulthood (Linsell et al. 2018; Breeman et al. 2016) and are associated with lower levels of education, employment, income and self-esteem (Saigal et al. 2016; Bilgin, Mendonca, and Wolke 2018). Preterm born adults have higher rates of internalising and avoidant personality problems than term born peers (Pyhälä

et al. 2017) and are less likely to form partner relationships or have children (Mendonça, Bilgin, and Wolke 2019).

Preterm birth is a global public health problem associated with a range of adverse lifecourse outcomes, significant health and education costs to society and a legacy that continues to impact future generations. There is an urgent need for effective treatments to reduce the risk of impairment. Further knowledge of the ontogeny and neurobiology underlying the brain dysmaturation associated with preterm birth is required to identify potential therapeutic targets.

1.2 Preterm brain injury

1.2.1 Overview of brain development

Neurodevelopment in utero represents a period of rapid brain growth (Andescavage et al. 2017; O'Rahilly and Muller 2006) with many complex dynamic processes vulnerable to injury associated with preterm birth, including neurogenesis, glial development and myelination. Key neurodevelopmental processes in humans are outlined in Figure 1-1.

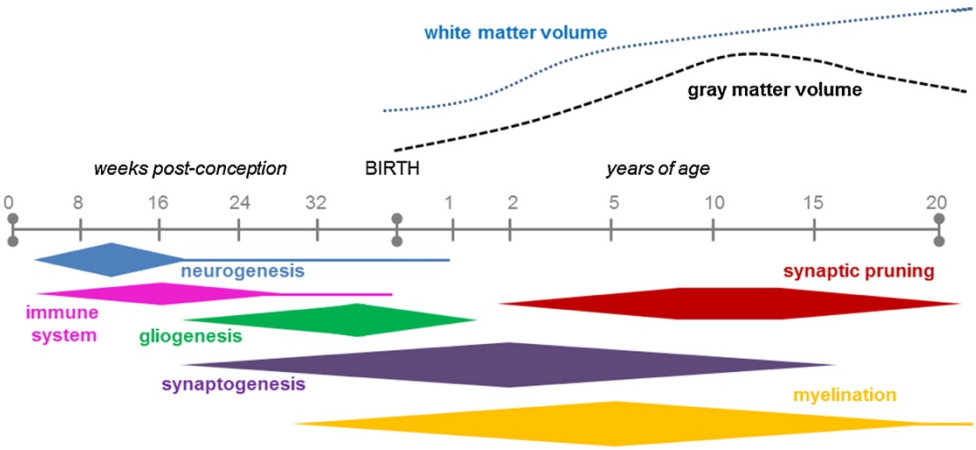

Figure 1-1 Key neurodevelopmental processes in humans.
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1.2.1.1 Neurogenesis

Neurogenesis begins shortly after neural tube formation at post-conceptional week (PCW) 4 (Bayer and Altman 2007).The wall of the neural tube contains a layer of neuroepithelial cells called the ventricular zone (VZ), where symmetrical division of neural progenitor cells (NPCs) expands the progenitor pool. The earliest neurons of the cortical plate emerge at PCW 7. The subventricular zone (SVZ) is established above the VZ and contributes to the generation of all excitatory projection neurons and glia via radial glia (RG) and transit amplifying intermediate progenitor cells (IPCs) (Kriegstein and Alvarez-Buylla 2009; Taverna, Gotz, and Huttner 2014; Silbereis et al. 2016).

1.2.1.2 Neuronal migration

Once formed neurons migrate from their site of origin in proliferative zones to the developing six-layered neocortex with the deepest layer populated first (Marin-Padilla 2014). By 24 weeks, the cerebral cortex has been populated by the full complement of glutamatergic excitatory projection neurons, whilst tangential migration of gamma-aminobutyric acid (GABA)-expressing interneurons from the ganglionic eminences occurs later, reaching peak density at term (Xu et al. 2011; Hansen et al. 2013; Arshad et al. 2016).

1.2.1.3 Organisation

On arrival at the cortical plate neurons continue to mature with a protracted period of neurite outgrowth, dendritic arborisation and synaptogenesis which continues into early childhood. The earliest synapses are formed in the subplate zone, which functions as a site of synaptic contact for ascending axonal afferents and provides axonal path guidance in cortical circuit formation (Kostovic and Judas 2010; Haynes et al. 2005; Kanold 2009). Development of the subplate peaks between PCW 24 and 32, a time of critical vulnerability for the preterm infant with injury likely to impact emerging cerebral connectivity (McQuillen and Ferriero 2005; Takahashi et al. 2012).

1.2.1.4 Gliogenesis

Astrocytes and oligodendrocyte precursors are generated from RG cells following neurogenesis (Howard et al. 2008), from PCW 30 to term. Astrocytes are multifaceted cells with important roles in development and homeostasis, providing nutritional, metabolic and structural support (Reemst et al. 2016).

Microglia originate from erythromyeloid precursor cells and infiltrate the CNS from PCW 4 (Lenz and Nelson 2018). Microglia are the resident macrophages of the brain involved in innate immunity and the maintenance of physiological homeostasis (Menassa and Gomez-Nicola 2018). Recently developed tools have demonstrated an array of microglial phenotypes with different context specific roles in neurodevelopment (Bennett et al. 2016). The major influx occurs at around PCW 16 but it is not until PCW 35 that they become widely distributed. Microglia are abundant in white matter in association with developing tracts (Verney et al. 2010; Billiards et al. 2006) and contribute to axonal development, oligodendrocyte maturation and myelination (Reemst et al. 2016; Hammond, Robinton, and Stevens 2018; Hagemeyer et al. 2017; Frost and Schafer 2016; Matsui and Mori 2018). If activated in response to danger signals, both astrocytes and microglia can release damaging pro-inflammatory cytokines, free radicals and glutamate, which may disturb key maturational events (Liddelow et al. 2017; Matsui and Mori 2018; Rothhammer and Quintana 2015).

1.2.1.5 Myelination

The process of myelination begins with oligodendrocyte progenitor cell (OPC) proliferation and differentiation. OPC's transition into pre-oligodendrocytes (pre-OL) with ongoing proliferative capacity and then post-mitotic immature oligodendrocytes (OL) before maturing to produce myelin.

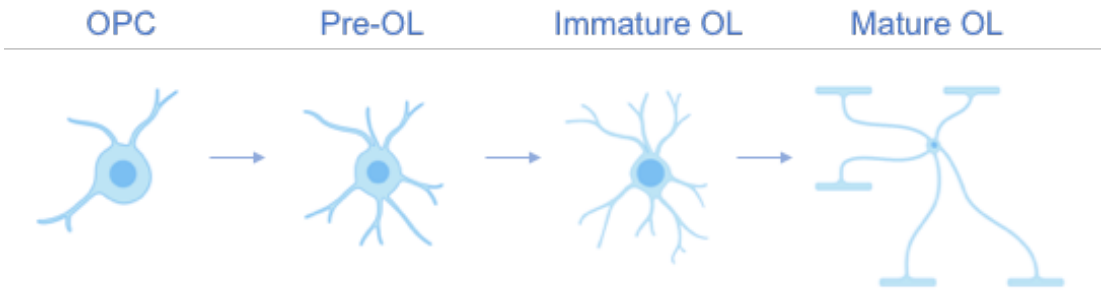

Figure 1-2. Oligodendrocyte maturation.

This transition results in a predominance of pre-oligodendrocytes in cerebral white matter from 24 PCW (Back et al. 2002). The initial deposition of myelin around axons progresses to form a more compacted myelin sheath. Whilst this process begins during the third trimester, mature myelin-producing oligodendrocytes are not abundant in white matter until after term (Semple et al. 2013).

Perturbation of these key developmental processes due to environmental exposures associated with preterm birth has multiple adverse consequences for the developing brain.

1.2.2 The encephalopathy of prematurity

Preterm brain injury is multifactorial and results in a combination of destructive lesions and developmental disturbances collectively described as the encephalopathy of prematurity (Volpe 2009).

Immature vasculature, cardiovascular instability and limited cerebral autoregulation predisposes to intraventricular and cerebellar haemorrhage. Intraventricular haemorrhage (IVH) typically occurs in the first 72 hours of life with bleeding into the germinal matrix followed by extension into the ventricular system. This can be categorised into four grades depending on site and severity (Papile et al. 1978) with grade IV representing a destructive parenchymal lesion resulting from haemorrhagic venous infarction (Bassan et al. 2006). Cerebellar haemorrhage ranges from mild punctate lesions to more extensive bilateral haemorrhages. The incidence of intraventricular and

cerebellar haemorrhage increases with the degree of prematurity and they often co-exist (Fumagalli et al. 2015; Limperopoulos, du Plessis, and Volpe 2018).

Preterm infants are at risk of cerebral hypoxia-ischaemia and infection/inflammation both in utero and postnatally during neonatal intensive care due to immature organ systems and the complications of prematurity. These processes trigger pathways of glutamate excitotoxicity, free radical injury and neuroinflammation. Activation of microglia and astrocytes results in the generation of pro-inflammatory cytokines and chemokines leading to altered blood brain barrier integrity, recruitment of peripheral immune cells and cellular injury (Baburamani et al. 2014; Back 2017).
Pre-oligodendrocytes, subplate neurons and developing axons are particularly susceptible to injury. This results in abnormal brain growth, white matter injury, impaired cortical and thalamic development, microstructural dysmaturation and altered connectivity of developing neural networks (Volpe 2019).

White matter is the predominant site of brain injury in the preterm population due to the vulnerability of pre-oligodendrocytes to injury and cell death (van Tilborg et al. 2018; Volpe 2019; Buser et al. 2012). Following insult, the population of pre-oligodendrocytes are replenished but they fail to differentiate. Arrested oligodendrocyte maturation results in hypomyelination and a spectrum of white matter injury, from diffuse axonal injury with gliosis to localised necrosis in cystic periventricular leukomalacia (PVL). However, white matter injury is also associated with grey matter neuronal loss and gliosis affecting the cortex, thalamus and basal ganglia (Pierson et al. 2007; Ligam et al. 2009). Damage to developing white matter axons and subplate neurons impairs cortical dendritic development and synaptogenesis with adverse consequences for developing neural networks (Sarnat et al. 2015). Furthermore, impaired interactions between the cortex and cerebellum

alongside exposure of the vulnerable proliferating external granule layer to damage mediators in the CSF also results in cerebellar growth failure.

1.3 Imaging the developing brain

1.3.1 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a safe, non-invasive technique which allows a detailed assessment of brain anatomy using structural and diffusion weighted imaging. Technical developments and processing pipelines optimised for fetal and neonatal imaging have generated a vast array of powerful tools to study the developing brain (Pecheva et al. 2018; Dubois et al. 2020; Hinojosa-Rodríguez et al. 2017). Most scans are now performed using a 1.5 or 3 Tesla scanner. Signal-to-noise ratio has been improved through the use of a close fitting neonatal head coil, protocols have been developed to optimise image resolution in the neonatal brain and several groups have demonstrated success in obtaining high quality images without the need for sedation.

1.3.2 Conventional structural MRI

T1-weighted (T1w) and T2-weighted (T2w) imaging provides contrast between tissues at millimetre resolution and can detect brain lesions associated with preterm birth at term-equivalent age. Ths includes recognised pathologies frequently identified on cranial ultrasound such as IVH, haemorrhagic parenchymal infarction and cystic PVL but also more subtle features such as diffuse white matter abnormalities and cerebellar haemorrhage.

Diffuse white matter abnormalities include diffuse excessive high signal intensity (DEHSI) and punctate white matter lesions (PWML) (Counsell, Allsop, and Harrison 2003; Bassi, Chew, and Merchant 2011). DEHSI is present in up to 80% of very preterm infants at term-equivalent age (Dyet et al. 2006), whilst PWML are observed in 10-24% of very preterm infants,

predominantly seen in the regions of the centrum semiovale, corona radiata and arcuate fasciculi (Neubauer et al. 2017; Tusor et al. 2017). PWML have high signal intensity on T1-weighted imaging and frequently low intensity on T2-weighted imaging suggesting an underlying ischaemic pathology or increased cellularity (Rutherford et al. 2010). Figure 1-3 shows T2-weighted images of common neonatal pathologies seen on conventional structural MRI in preterm infants at term-equivalent age.

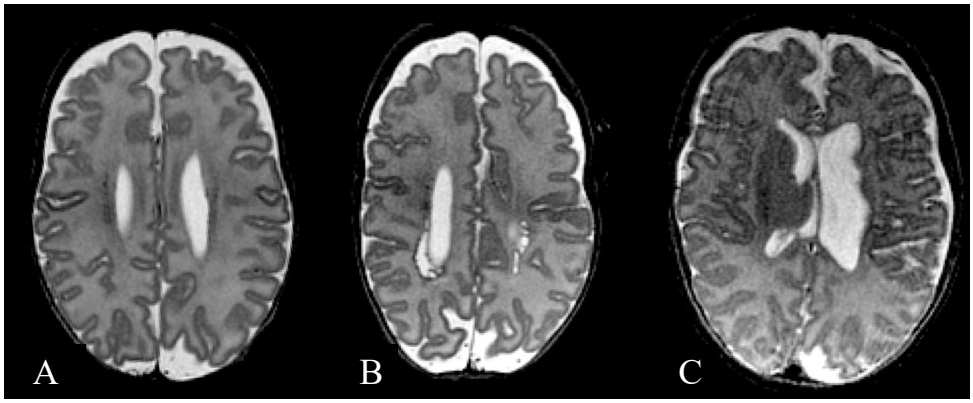

Figure 1-3. Transverse T2-weighted images at term-equivalent age showing (A) punctate white matter lesions, (B) periventricular leukomalacia, and (C) haemorrhagic parenchymal infarction.
Adapted from (Plaisier et al. 2014) with permission.

Severe destructive lesions such as cystic PVL and haemorrhagic parenchymal infarction are associated with major disability such as CP, whilst PWML and cerebellar lesions are more often associated with cognitive and behavioural problems (Hintz et al. 2018; Villamor-Martinez, Fumagalli, et al. 2019; Hortensius et al. 2018; Arulkumaran et al. 2020; Woodward et al. 2012; Mukerji, Shah, and Shah 2015; Guo et al. 2017).

However, the prognostic ability of structural MRI is modest. A recent study of 504 very preterm infants showed that 3T MRI at term-equivalent age with no focal lesion has a sensitivity of 45% (95% CI, 40-50%), and a specificity of 61% (95% CI, 51%-71%) for normal neurodevelopmental outcome (Arulkumaran et al. 2020).

1.3.3 Quantitative structural MRI

Quantitative techniques can be applied to structural images to enable brain tissue segmentation and anatomical parcellation of regions of interest. Computational approaches have shown that preterm infants have significant abnormalities in regional brain growth and development when compared to term-born controls.

Morphometric studies have shown that preterm infants have reduced total and regional brain volumes affecting the white matter, thalamus, basal ganglia, hippocampus, cortex and cerebellum (Padilla et al. 2014; Makropoulos et al. 2016; Inder et al. 2005; Boardman et al. 2006; Thompson et al. 2007; Strahle et al. 2019).

Studies have also demonstrated altered cortical folding and reduced cortical surface area in preterm infants when compared to term-born controls (Shimony et al. 2016; Engelhardt et al. 2015). The fastest growing brain tissues between preterm birth and term-equivalent age are the cortical gray matter and cerebellum and longitudinal measurements are predictive of cognitive and motor outcomes in early childhood (Rathbone et al. 2011; Moeskops et al. 2017; Gui et al. 2019).

These imaging signatures of neuronal loss, axonal disturbance and altered gyrification are present at term-equivalent age but also persist into childhood and adolescence (Zhang et al. 2015; Nosarti et al. 2002; Papini et al. 2020).

More advanced quantitative MRI techniques to assess brain microstructure provide an early indicator of cell damage and have further enhanced our understanding of the neurobiology underlying these imaging signatures.

1.3.4 Diffusion tensor imaging

Diffusion tensor imaging (DTI) is an advanced MRI technique which enables assessment of microstructural characteristics through the measurement of

water diffusion in brain tissue. DTI measures the magnitude and direction of water molecule diffusion along multiple axes to obtain a mathematical representation of water movement within individual voxels of an image (Basser, Mattiello, and LeBihan 1994). Measurements acquired from multiple directions can be used to generate a tensor matrix which can be analysed to produce three eigenvalues (λ_1, λ_2, λ_3) and three eigenvectors (ε_1, ε_2, ε_3). The primary eigenvalue (λ_1) represents water diffusivity along the principal axis (ε_1), known as axial diffusivity (AD). Radial diffusivity (RD) is calculated from the average of the second and third eigenvalues (λ_2, λ_3) and describes the average water diffusion perpendicular to the principal axis. Mean diffusivity (MD) is the average of all 3 eigenvalues and describes the magnitude of water diffusion within a voxel.

Fractional anisotropy (FA), a measurement that quantifies the shape of the diffusion ellipsoid, is a scalar taking values from 0 to 1 which represents the degree of directional variation of water diffusion. In the CSF, water diffusion is unrestricted or isotropic with eigenvalues equivalent in all directions (FA = 0), creating a spherical ellipsoid. Within brain tissue, water diffusion is directionally dependent due to hindrance by cellular membranes with diffusion anisotropy closer to 1 (FA ~ 0.5 to 0.8) (Pierpaoli and Basser 1996; Beaulieu 2002).

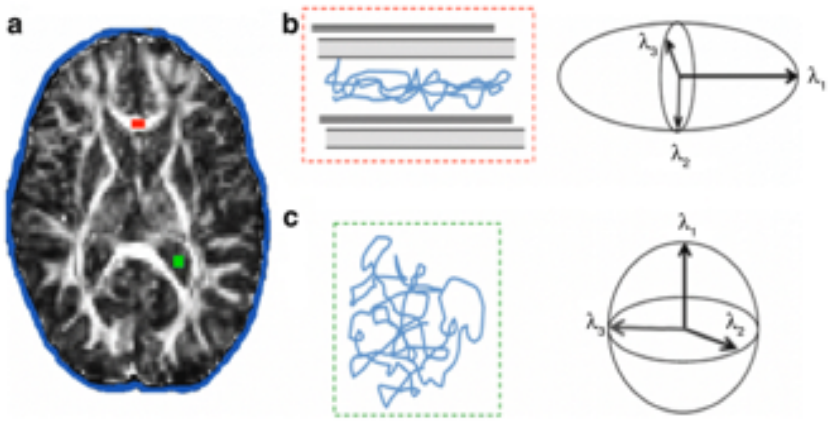

Figure 1-4. Isotropic and anisotropic water diffusion in the brain.
In the white matter of the corpus callosum (shown in red), water diffusion occurs preferentially along axonal tracts, known as anisotropic diffusion (b). In the CSF (shown in green), water diffusion is isotropic (c)
Reproduced with permission from (Pandit et al. 2013), Copyright Springer Nature.

FA is sensitive to microstructural changes including axonal size and density, AD is thought to describe axonal integrity whilst RD provides information about oligodendrocyte ensheathment of axons and later myelination (Pandit et al. 2013). Representative images of FA, MD, AD and RD in the neonatal brain are shown in Figure 1-5.

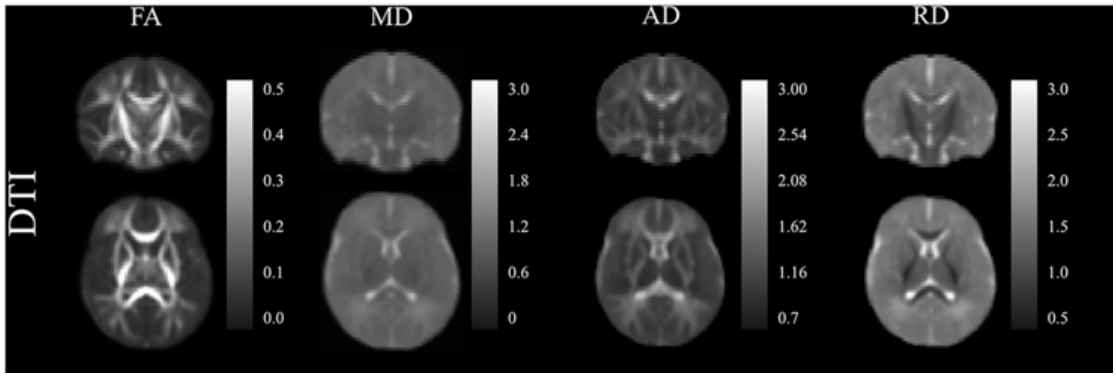

Figure 1-5. Coronal and axial slices demonstrating water diffusion tensor maps in the neonatal brain.
Reproduced with permission from (Dean et al. 2017).

The quantitative information from DTI has been used to describe the characteristic microstructural changes associated with early brain development. White matter maturation is associated with increasing FA and reductions in MD and RD, reflecting microstructural changes associated with axonal packing, membrane proliferation and myelination (Huppi et al. 1998; Kersbergen et al. 2014; Dubois et al. 2014), shown in Figure 1-6.

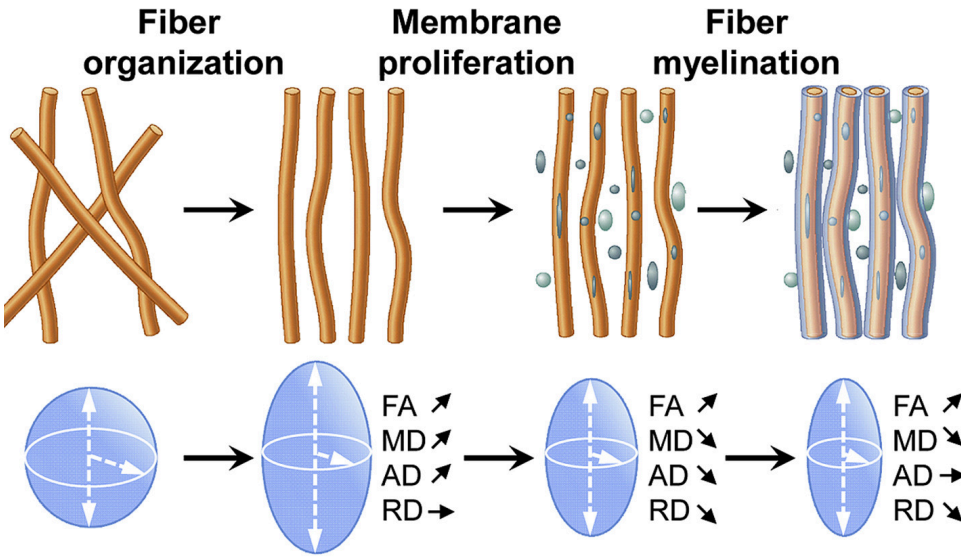

Figure 1-6. The relationships between maturational processes and water diffusion metrics in white matter.
Reproduced from (Ouyang et al. 2019) with permission.

In the cortex, maturation is characterised by decreasing FA and MD reflecting dendritic arborisation and neurite growth (Batalle et al. 2019; Ouyang et al. 2019; Bouyssi-Kobar et al. 2018).

By term-equivalent age, the preterm brain is structurally different from that of healthy term-born controls, characterised by lower FA and higher MD in white matter (Anjari et al. 2007; Rose et al. 2008). This is exacerbated by exposure to cumulative risk factors such as fetal growth restriction, bronchopulmonary dysplasia and necrotising enterocolitis, and is associated with neurodevelopmental performance in early childhood (Counsell et al. 2008; van Kooij et al. 2012; Ullman et al. 2015; Barnett et al. 2018).

Whilst water diffusion parameters demonstrate microstructural alterations, the DTI model lacks tissue specificity and can be inaccurate when there are multiple fibre populations or when fibres cross within an individual voxel (Tournier, Mori, and Leemans 2011). High angular resolution diffusion imaging (HARDI) data can be used to apply more complex biophysical models, such as neurite density and orientation dispersion index (NODDI) to describe microstructural complexity characterised by the degree of diffusion

restriction within different compartments (Zhang, Schneider, et al. 2012). NODDI models the intracellular, extracellular and CSF fractions to calculate a neurite density index (NDI) and an orientation dispersion index (ODI) which describes both cellular density and the geometrical organisation of neurites. Representative images of the volume fraction of the intracellular compartment (v_{IC}), volume fraction of the isotropic diffusion compartment (v_{ISO}) and ODI in the neonatal brain are shown in Figure 1-7.

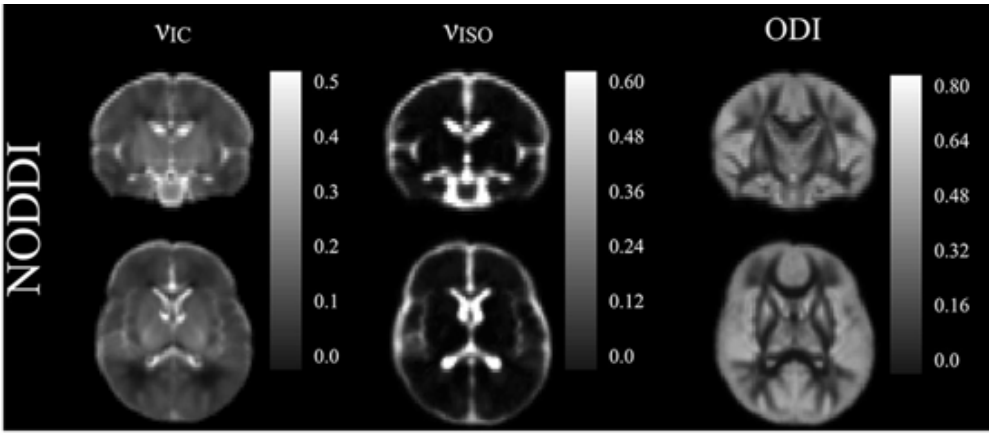

Figure 1-7. Coronal and axial slices demonstrating NODDI maps in the neonatal brain. Reproduced with permission from (Dean et al. 2017).

Perinatal maturation is characterised by increasing NDI in white matter and increasing ODI in grey matter and both are affected by prematurity (Kunz et al. 2014a; Eaton-Rosen et al. 2015; Batalle et al. 2019).

The integration of DTI scalars and NODDI metrics in morphometric similarity networks (Galdi et al. 2020; Fenchel et al. 2020) and histogram based analyses (Blesa et al. 2020) have recently been optimised for studying the neonatal brain.

Peak width of skeletonised mean diffusivity (PSMD) is a method for histogram-based calculation of MD distribution across the entire white matter skeleton, which provides a single measure of generalised white matter microstructure. PSMD has been used to describe white matter disease burden in the context of multiple sclerosis and cerebrovascular disease and is strongly associated with processing speed and cognitive performance

(Baykara et al. 2016; Deary et al. 2019; Wei et al. 2019; Low et al. 2020). The histogram model was recently extended to include 5 other histogram-based metrics derived from DTI and NODDI to evaluate their accuracy as biomarkers of white matter dysconnectivity associated with preterm birth. Whilst there were group differences in peak width of skeletonised axial and radial diffusivities (PSAD, PSRD) and orientation dispersion index (PSODI) in preterm infants when compared to term-born controls, peak width of skeletonised neurite density index (PSNDI) and PSMD were found to be the best classifiers of gestational age at birth with an accuracy of 81±10% and 77±9% respectively (Blesa et al. 2020). As sensitive markers of generalised white matter connectivity in the developing brain, PSMD and PSNDI offer promising tools to investigate the effect of perinatal exposures on developing microstructure. The imaging pipeline for the generation of PSMD and PSNDI is described in Chapter 2, section 2.5.5.

1.4 The role of inflammation in preterm brain injury

1.4.1 The innate immune response

Inflammation is a complex response to infection or tissue injury involving the interaction between cells and a large range of soluble factors. Pattern recognition receptors (PRRs) on mast cells and macrophages recognise pathogen associated molecular patterns (PAMPs) from invading microbes or damage-associated molecular patterns (DAMPs) from damaged cells to generate a variety of inflammatory mediators including cytokines and chemokines alongside activation of the complement cascade. Pro-inflammatory cytokines Interleukin (IL)-1β, IL-6 and Tumor necrosis factor-α (TNF-α) elicit an acute phase response co-ordinated by the liver through the release of regulatory proteins such as C-reactive protein (CRP). Chemokines including IL-8, Monocyte chemotactic protein-1 (MCP-1), and Regulated upon activation, normal T cell expressed and secreted (RANTES), facilitate the recruitment of neutrophils to the site of infection or injury through chemotaxis and endothelial activation (Turner et al. 2014). On arrival,

neutrophils become activated and attempt to clear invading pathogens by phagocytosis or toxic degranulation.

The complement system is an important branch of the innate immune system comprising multiple recognition molecules which when activated, result in the recruitment of a cascade of enzymes to produce potent anaphylatoxins that enhance phagocytosis and the lytic membrane attack complex (MAC). Ordinarily, the combined mechanisms of the innate immune response facilitate pathogen clearance and tissue repair but when dysregulated can result in an amplified response and tissue damage (Medzhitov 2008; Nathan 2002).

1.4.2 Chorioamnionitis and the fetal inflammatory response

One example of perinatal immune activation in preterm infants is histologic chorioamnionitis (HCA). HCA describes intrauterine inflammation, characterised by the infiltration of neutrophils into maternal or fetal placental structures (Redline et al. 2003). HCA complicates 40-70% of preterm births with a higher incidence associated with prolonged rupture of membranes (PROM), spontaneous preterm labour and lower gestational age (Goldenberg et al. 2008; Stoll et al. 2010). Whilst intraamniotic inflammation in the absence of infection can occur (Romero et al. 2015), the most frequent cause is ascending invasion by common microbes of the lower genital tract such as Ureaplasma, Group B Streptococcus, Escherichia coli and Fusobacteria (DiGiulio et al. 2008; Yoneda et al. 2016; Leviton et al. 2010). The amniotic sac is composed of the transformed maternal endometrium of pregnancy (the decidua) in direct contact with fetal components. In response to a chemotactic gradient, neutrophils migrate from the maternal capillaries of the decidua into the fetal chorion and then amniotic membranes, termed chorioamnionitis. The fetal inflammatory response is characterised by inflammation involving the vessels of the chorionic plate, known as vasculitis, or the umbilical cord, termed funisitis. Acute funisitis begins as inflammation of the umbilical vein (phlebitis) followed by involvement of umbilical arteries (arteritis) in multiple foci which merge as inflammation becomes more

advanced. (Kim et al. 2015; Presicce et al. 2018). Figure 1-8 shows placental anatomy and possible sites of inflammation in the context of intrauterine infection.

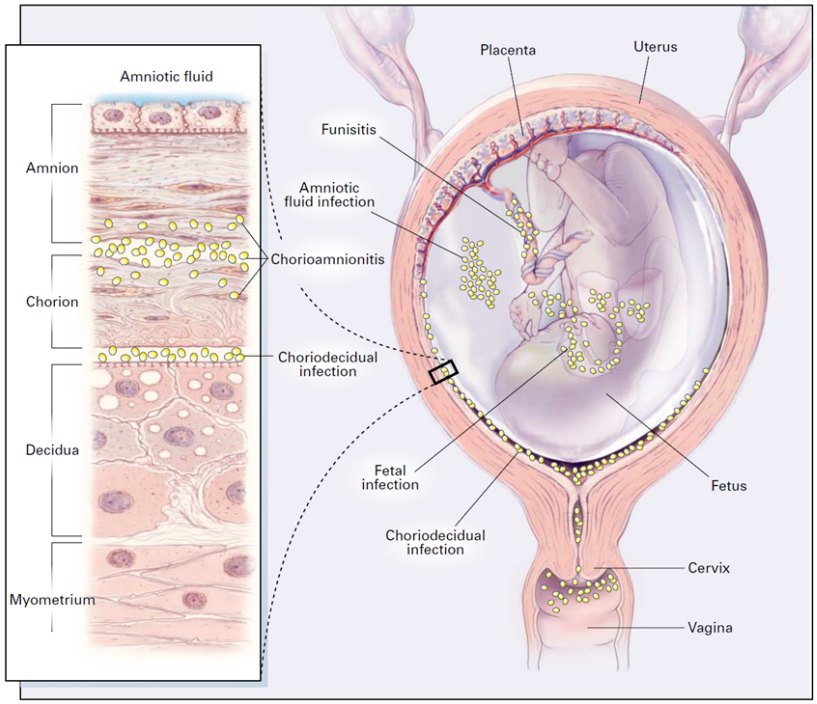

Figure 1-8. Placental anatomy in the context of intrauterine infection. Reproduced with permission from (Goldenberg, Hauth, and Andrews 2000), Copyright Massachusetts Medical Society.

The most frequently used staging system classifies acute inflammatory placental lesions into maternal inflammatory responses and fetal inflammatory responses with high reproducibility (Redline et al. 2003). The severity of funisitis has been correlated with fetal plasma IL-6 (Kim et al. 2001), which is a major mediator of the acute systemic inflammatory response. Fetal inflammatory response syndrome (FIRS) was initially defined as umbilical cord blood IL-6 >11ng/mL (Gomez et al. 1998; Gotsch et al. 2007; Pacora et al. 2002) but subsequent studies have shown that the fetal inflammatory response is associated with alterations in a wide range of pro-inflammatory mediators (Hecht et al. 2011).

1.4.3 Neuroinflammation in the developing brain

Activation of the systemic inflammatory response results in the generation of a complex network of immune mediators capable of initiating a neuroinflammatory response in the developing brain (Hagberg et al. 2015). Preterm birth is associated with a distinct CSF inflammatory profile (Boardman et al. 2018) and post-mortem studies show upregulated expression of several pro-inflammatory cytokines in the brains of infants with confirmed infection (Kadhim et al. 2001; Folkerth et al. 2004). Peripheral immune mediators may access the CNS due to altered blood brain barrier permeability (Stolp et al. 2005), or may act via receptors expressed in the circumventricular organs, choroid plexus and leptomeninges (Rivest 2003; Galea, Bechmann, and Perry 2007). However, activation of resident microglia plays a key role in the pathogenesis of CNS inflammation and brain injury (Mallard, Tremblay, and Vexler 2019).

Microglia express a range of PRRs which can become activated by damaged tissues, cytokines, bacterial products or invading organisms (Kettenmann et al. 2011). Activated microglia secrete a range of soluble factors to propagate the immune response and undergo morphological changes to facilitate phagocytosis of dead cells and debris. The vast array of cytokines and chemokines produced promote microglial proliferation, induce pro-inflammatory reactive astrocytes, and function as chemo-attractants for other circulating immune cells (Liddelow et al. 2017). Increased production of cell adhesion molecules facilitates migration and extracellular matrix proteinases, such as matrix metalloproteinases (MMPs) contribute to breakdown of the blood brain barrier. Pro-inflammatory cytokines and chemokines contribute to glutamate excitotoxicity and free radical injury when produced by microglia in excess, causing neuronal cell death, astrogliosis and pre-oligodendrocyte maturation arrest (Baburamani et al. 2014).

Neuroinflammation can also activate the adaptive immune response, which may contribute to the pathogenesis of brain injury by shifting naïve helper T

(Th) cells patrolling the borders of the CNS towards a pro-inflammatory Th1 differentiation and via inhibition of regulatory T cells (Leviton, Dammann, and Durum 2005; Vivier and Malissen 2005; Carroll 2004; Ellwardt et al. 2016; Nguyen, Julien, and Rivest 2002). CD3+ T cells and CD 20+ B cells have been observed in the post-mortem brains of preterm infants with white matter injury (Nazmi et al. 2018).

Neuroinflammation results in injury to subplate neurons, migrating white matter axons and pre-oligodendrocytes during critical phases of white matter development and cortical plasticity with long-term adverse consequences on brain microstructure, connectivity and function.

1.4.4 Outcomes following perinatal inflammation

Preterm infants exposed to HCA in utero are at increased risk of developing neonatal complications, including respiratory distress syndrome (RDS), bronchopulmonary dysplasia **(**BPD), early-onset sepsis (EOS), necrotising enterocolitis (NEC) and retinopathy of prematurity (ROP) (Bose et al. 2011; Villamor-Martinez, Álvarez-Fuente, et al. 2019; Been et al. 2013; Villamor-Martinez et al. 2018; Hofer et al. 2013; Ozalkaya et al. 2016; Otsubo et al. 2017).
HCA has also been associated with white matter injury (Anblagan et al. 2016), cerebral palsy (Shatrov et al. 2010; Shi et al. 2017), neurodevelopmental impairment and autism spectrum disorder (Al-Haddad et al. 2019; Salas et al. 2013; Venkatesh et al. 2020).

Similar associations have been observed between neonatal infection and neurocognitive performance in childhood (Stoll et al. 2004; Schlapbach et al. 2011; Bassler et al. 2009; Pappas et al. 2014; Francis et al. 2019; Salas et al. 2013; Bright et al. 2017), with repeated episodes of inflammation increasingly recognised to confer additive risks to the developing brain (O'Shea et al. 2012; O'Shea et al. 2013; Hansen-Pupp et al. 2008; Kuban et al. 2014; Leviton et al. 2016; Yanni et al. 2017; Kuban et al. 2017; Barnett et al. 2018).

Fetal exposure to inflammation may influence innate and adaptive immune programming which may explain this multi-hit hypothesis (Sabic and Koenig 2020). Whole blood transcriptomic analyses showed that 488 genes were differentially expressed in the context of HCA (Weitkamp et al. 2016), including microRNA 155 (miR-155), a known master regulator of immune responses (Mahesh and Biswas 2019). HCA has been associated with the upregulation of pro-inflammatory T helper cell subsets, such as Th17 cells (Rito et al. 2017) which can secrete IL-17 and may contribute to organ injury (Lawrence and Wynn 2018) and RNA sequencing of HCA exposed monocytes showed an altered responsiveness to subsequent inflammatory stimulation in vitro (de Jong et al. 2018; Bermick et al. 2019). Altered immune programming in utero may therefore contribute to an increased risk of subsequent infection and/or dysregulation of immune responses to future inflammatory exposures.

Specific inflammatory mediators have been investigated as potential biomarkers of HCA and long-term outcomes. Higher levels of IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α in umbilical cord blood, postnatal blood, and CSF have been associated with the development of IVH and white matter injury after preterm birth (Duggan et al. 2001; Lu et al. 2016; Ellison et al. 2005; Leviton et al. 2018; Hansen-Pupp et al. 2005) whilst higher umbilical cord blood and postnatal blood levels of TNF-α, IL-8 and IL-18 have been associated with the development of CP (Hansen-Pupp et al. 2008; Yanni et al. 2017; Minagawa et al. 2002; Carlo et al. 2011).
Elevated levels of IL-8, TNF-α, and ICAM-1 in the first month of life were associated with increased risk of executive dysfunction (Leviton et al. 2019) amongst preterm born children whilst higher circulating neurotrophins were associated with a reduced risk of cognitive impairment (Kuban et al. 2018).

1.5 Experimental models of preterm brain injury

1.5.1 Animal models

Experimental models of preterm brain injury in a range of different species have consistently shown that exposure to gram-negative endotoxin lipopolysaccharide (LPS) or systemic administration of IL-1β causes microglial activation and neuroinflammation with resulting grey and white matter damage (Boksa 2010; Kuypers et al. 2012; Favrais et al. 2011; Mallard, Tremblay, and Vexler 2019; Smith, Hagberg, et al. 2014; Dean et al. 2011; Dieni et al. 2004).

Whilst the underlying mechanisms linking microglial activation with brain dysmaturation are not well understood, several studies suggest that pro-inflammatory cytokines are likely mediators of injury (Pang, Cai, and Rhodes 2003; Nobuta et al. 2012; Bonestroo et al. 2015; Serdar et al. 2019). Activation of the innate immune system via toll-like receptors (TLR) (Lalancette-Hébert et al. 2017) also makes the developing brain more susceptible to future insults (Eklind et al. 2001). TLR activation leads to the production of pro-inflammatory cytokines IL-1β and TNF-α via the MyD88 pathway. Inhibition of downstream MyD88 pathways which reduce NF-κB and JNK signalling have been shown to reduce microglial activation, neuroinflammation and cellular injury (Wang et al. 2009; Yang et al. 2013).

Although animal models can recapitulate some of the features of the encephalopathy of prematurity, the white matter injury described is characterised by destructive lesions and hypomyelination rather than the more commonly encountered diffuse white matter abnormalities and the effects of neuroinflammation on grey matter development is less well studied. The timing, location and severity of insult are likely to be important in the pathogenesis of brain dysmaturation after preterm birth and sustained inflammation is a recognised feature of the neonatal immune response (Skogstrand et al. 2008; Dammann and Leviton 2014). Species differences in

the neurobiology of key brain maturation events can therefore make human comparisons to animal models difficult to interpret (Hodge et al. 2019; Gussenhoven et al. 2018).

1.5.2 Primary human tissue

Neuropathological studies have highlighted the role of microglial activation, oxidative stress and nitrosative injury in the pathogenesis of white matter disease and associated grey matter abnormalities including diffuse axonal injury and reduced cortical density (Haynes et al. 2008; Haynes et al. 2009; Haynes and van Leyen 2013; Andiman et al. 2010). However, post-mortem analysis provides only a single reading with little insight into the preceeding developmental trajectory.

Dissociated cells or organotypic slice cultures using fetal tissues have advanced understanding of progenitor cell behaviour and cortical development (Subramanian et al. 2017; Mayer et al. 2019) but access to human fetal brain tissue is limited and cultures cannot be maintained long-term, limiting opportunities for experimental manipulations.

1.5.3 Modelling neurodevelopment using iPSCs

The use of induced pluripotent stem cells (iPSCs) reprogrammed from human somatic cells offer the opportunity to study specific aspects of human neurodevelopment *in vitro*.

Takahashi et al. first generated human induced pluripotent stem cells (iPSCs) through the viral transduction of adult human fibroblasts into a pluripotent state using four transcription factors: Oct3/4, Sox2, c-Myc and Klf4 (Takahashi et al. 2007). iPSCs have the ability to self-renew indefinitely and form any cell type in the human body under the right conditions.

iPSCs reprogrammed towards neuroectoderm can be differentiated into neural progenitor cells and then specific neuronal sub-types with the addition

of growth factors and mitogens in chemically defined medium. Protocols have been established for the efficient generation of a highly enriched population of mature post-mitotic glutamatergic excitatory neurons capable of spontaneous and induced depolarisation and the formation of functional synapses (Stein et al. 2014; Bilican et al. 2014; Livesey et al. 2016). This monolayer system can be used to study the impact of environmental exposures on key processes of human neural development *in vitro* using quantifiable measures of neuronal maturation and morphology.

Human iPSC models have advanced understanding of the cellular and molecular mechanisms underlying neurodevelopmental disorders with specific gene mutations but have also been applied to the study of complex disorders which result from a combination of genetic susceptibility and environmental exposure such as schizophrenia and ASD (Ardhanareeswaran et al. 2017). iPSC models of ASD have identified dysregulation of genes involved in cell proliferation, neuronal differentiation and synaptogenesis suggesting a selective vulnerability in early cortical development (Mariani et al. 2015; Schafer et al. 2019). ASD is more prevalent in children who were born preterm and is also associated with neuroinflammation (Matta, Hill-Yardin, and Crack 2019), suggesting that immune dysregulation may lead to a spectrum of neurodevelopmental disorders with common underlying neurobiology.

iPSC-derived cortical neurons have a phenotype and transcriptomic profile resembling fetal brain development (Stein et al. 2014; Livesey et al. 2016), making this scalable system a promising tool for the study of preterm brain injury and the impact of specific inflammatory mediators on early cortical development.

1.6 Aims and hypotheses

Perinatal inflammation is associated with an increased risk of neonatal morbidity and a range of adverse neurodevelopmental outcomes. However, the immune mechanisms underlying these associations remain poorly understood. Studies investigating the association of specific inflammatory mediators with imaging features of brain injury have so far been limited to cranial ultrasound and conventional structural MRI and outcomes assessed beyond the neonatal period are subject to environmental confounders. This thesis aims to identify specific immune mediators associated with atypical brain development after preterm birth by addressing three main hypotheses:

I. (a) The umbilical cord blood profile of preterm infants will be pro-inflammatory when compared to term-born controls (b) Specific inflammation-associated proteins in umbilical cord blood will be predictive of histologic chorioamnionitis in preterm infants (c) The blood immune profile on postnatal day 5 will be altered in preterm infants who were exposed to histologic chorioamnionitis.

II. Specific inflammation-associated proteins in postnatal blood taken on day 5 will be associated with white matter microstructure in preterm infants characterised by diffusion MRI metrics (PSMD and PSNDI) at term-equivalent age.

III. Specific proteins associated with systemic inflammation after preterm birth will be associated with altered neuronal maturation and morphology in a human iPSC-derived model of cortical neuronal development.

2 Materials and methods

2.1 Patient recruitment

2.1.1 Participants

Participants were recruited to the Theirworld Edinburgh Birth Cohort Study, a single-centre longitudinal study of preterm birth with detailed biosampling and brain imaging from birth to 5 years (Boardman et al. 2020).

2.1.2 Ethics

Ethical approval was obtained from the UK National Ethics Service (South East Scotland Research Ethics Committee 16/SS/0154) and parents provided written informed consent.

2.1.3 Inclusion and exclusion criteria

Infants were delivered at the Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, UK between February 2017 and April 2019. Cases were infants delivered before 32 weeks of pregnancy based on first trimester ultrasound, while controls were healthy term-born infants delivered after 37 weeks. Infants with major congenital anomaly or contra-indications to MRI were excluded. Term-born infants who required admission to the neonatal unit were also excluded.

2.1.4 Recruitment

I led study recruitment with assistance from Clinical Research Fellow, Dr. David Stoye, and Research midwife, Gillian Lamb. Potential participants were identified using electronic patient records at the Royal Infirmary of Edinburgh. Antenatal consent was obtained where possible to enable collection of cord blood and placenta at delivery. Postnatal consent was obtained for infants if expedient delivery or clinical condition did not allow antenatal approach.

Consent to the study was gained in two stages: (1) for perinatal and neonatal sampling including brain MRI, and (2) for assessments following MRI to the age of 5 years.

To generate a control group representative of the general population with regard to mode of delivery and SIMD, term-born participants were recruited from a variety of sources including admissions for induction of labour, elective caesarean section lists and community outpatient clinics throughout NHS Lothian.

During the study period, the parents of 339 term infants were approached and 85 (25%) were recruited. Of the 188 eligible preterm infants admitted to the neonatal unit, all were approached and 153 (81%) were recruited.

2.2 Clinical data collection

2.2.1 Data collection procedure

Antenatal records were used to identify maternal age, parity, body mass index (BMI), maternal medical history, complications of pregnancy, mode of delivery, and exposure to antenatal steroids and magnesium sulphate. Neonatal electronic patient records were used to identify gestational age at delivery, sex, birthweight, bronchopulmonary dysplasia (BPD), early onset sepsis (EOS), late onset sepsis (LOS), meningitis, necrotising enterocolitis (NEC) and retinopathy of prematurity (ROP).

2.2.2 Definitions

Bronchopulmonary dysplasia (BPD) The need for supplemental oxygen or respiratory support at 36+0 weeks gestational age.

Early-onset sepsis (EOS) Onset before 72 hours of life. Positive blood culture and/or antibiotic treatment for at least 5 days.

Late-onset sepsis (LOS) Onset after 72 hours of life. Positive blood culture and/or antibiotic treatment for at least 5 days.

Necrotising enterocolitis (NEC) Medical treatment for 7 days or more or surgical treatment.

Prolonged rupture of membranes (PROM) Rupture of membranes >24 hours prior to delivery.

Retinopathy of Prematurity (ROP) Requiring treatment with laser therapy or anti-VEGF.

2.3 Dried blood spot collection

2.3.1 Collection procedure

Dried blood spot samples (DBSS) were taken from the umbilical cord following delivery for both preterm cases and term-born controls. For preterm infants, additional samples were collected on day 5 of life if a clinical sample was indicated. DBSS were obtained using a FTATM DMPK-A Card (Whatman^{TM} GE Healthcare).

2.3.2 Processing and storage

Bloodspot cards were dried at room temperature and then stored at -20° C until analysis at the Statens Serum Institut (Center for Neonatal Screening, Copenhagen, Denmark) which was performed by Dr. Nis Borbye-Lorenzen and Dr. Kristin Skogstrand.

2.3.3 Immunoassay

A customised multiple sandwich immunoassay based on flowmetric Meso-Scale technology was used to measure blood spot levels of Interleukin(IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, Monocyte chemotactic protein-1 (MCP-1), Macrophage inflammatory protein-1α (MIP-1α), Macrophage inflammatory protein-1β (MIP-1β), Tumor necrosis factor-α (TNF-α), Tumor necrosis factor-β (TNF-β), Brain-derived neurotropic factor (BDNF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon-γ (IFN-γ), C-reactive protein (CRP), matrix-metalloproteinase 9 (MMP-9), Regulated upon activation, normal T cell expressed and secreted (RANTES) and Complement components C3, C5a and C9.

Two 3.2 mm disks from the DBSS were punched into each well of Nunc 96-well polystyrene microwell plates (#277143, Thermo Fisher Scientific). 130 µl extraction buffer (PBS containing 1% BSA (Sigma Aldrich #A4503), 0.5% Tween-20 (#8.22184.0500, Merck Millipore), and complete protease inhibitor cocktail (#11836145001, Roche Diagnostics) was added to each well, and the samples were incubated for 1 hour at room temperature on a microwell shaker set at (900 rpm). The extracts were analyzed using U-plex plates (Meso-Scale Diagnostics (MSD), Maryland, US) coated with antibodies specific for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-17, TNF-α, MIP-1β on one plate (#K15067 customized) and BDNF, GM-CSF, IL-10, IL-18, IFN-γ, TNF-β, MCP-1, MIP-1α on another (#K151AC customized) (both MSD).

Supplier's instructions were followed, and extracts were analysed undiluted. A third multiplex analysis was developed in-house applying extracts diluted 1:10 in diluent 7 (#R54BB, MSD) using antibodies specific for C3 (HYB030-07 and HYB030-06, SSI Antibodies, Copenhagen, Denmark), C5a (10604-MM04 and 10604-MM06, Sino Biological, Eschborn, Germany), C9 (R-plex kit #F21XZ, MSD), MMP-9 (BAF911 and MAB911), RANTES (MAB278 and AF278NA) and CRP (BAM17072 and MAB1701) (all R&D Systems, Minneapolis, US) for coating the U-plex plate and for detection, respectively. Coating antibodies (used at 1 µg/mL, except CRP used at 10 ng/mL) were biotinylated (using EZ-Link Sulfo-NHS-LC-Biotin #21327, Thermo Fisher Scientific) in-house (if not already biotinylated at purchase) and detection antibodies were SULFO-tagged (R91AO, MSD), both at a challenge ratio of 20:1.

The following calibrators were used: C3: #PSP-109 (Nordic Biosite, Copenhagen, DK), C5a: #10604-HNAE (Sino Biological), C9: #F21XZ (from R-plex kit, MSD), MMP-9: #911-MP, RANTES: #278-RN and CRP: #1707-CR/CF (all from R&D Systems). Calibrators were diluted in diluent 7, detection antibodies (used at 1 µg/mL, except CRP used at 100 ng/mL) were

diluted in diluent 3 (#R50AP, MSD). Controls were made in-house from part of the calibrator solution in one batch, aliquoted in portions for each plate and stored at -20°C until use. The samples were prepared on the plates as recommended from the manufacturer and were immediately read on the QuickPlex SQ 120 (MSD). Analyte concentrations were calculated from the calibrator curves on each plate using 4PL logistic regression using the MSD Workbench software.

Intra-assay variations were calculated from 16 measurements of a pool of the same control sample on the same plate. Inter-assay variations were calculated from controls analysed in duplicate on each plate during the sample analysis, 4 plates in total. Limits of detection were calculated as 2.5 standard deviations from duplicate measurements of the zero calibrator. The higher detection limit was defined as the highest calibrator concentration. Median intra-assay variation was 8.2% and median inter-assay variation was 11.1%. Table 2-1 shows the DBSS detection limits and assay variations.

Analyte	Lower detection limit (pg/mL)	Higher detection limit (pg/mL)	Intra-assay CV%	Inter-assay CV%
BDNF	1.05	41000	2.71	3.84
C3	13739	50000000	3.62	15.20
C5a	544	16700000	5.60	19.10
C9	8.38	3300000	5.60	12.80
CRP	89.0	100000000	16.70	34.50
GM-CSF	0.101	12500	4.19	7.86
IFN-γ	0.646	34875	3.24	6.29
IL-1β	0.0260	4788	11.90	10.80
IL-2	0.340	2688	11.30	14.70
IL-4	0.00956	2700	16.10	16.80
IL-5	0.125	5125	12.00	9.67
IL-6	0.452	2650	11.10	20.9
IL-8	0.0684	2575	10.9	15.0
IL-10	0.0886	4625	4.13	5.56
IL-12p70	0.157	9063	10.30	17.00
IL-17	0.286	47375	13.50	11.40
IL-18	0.199	50500	3.36	6.58
MCP-1	1.28	7400	2.55	6.99
MIP-1α	0.960	7500	2.71	6.32
MIP-1β	1.73	2325	13.8	5.39
MMP-9	24.1	5000000	6.29	25.10
RANTES	37.9	1600000	10.10	29.00
TNF-α	0.272	4538	11.80	4.48
TNF-β	0.0250	5050	2.52	3.90

Table 2-1. DBSS detection limits and assay variations.

2.4 Placental histopathology

2.4.1 Collection and storage

After delivery, placentae were formalin fixed and stored at 4°C. Distal and proximal sections of the umbilical cord, a roll of extraplacental membranes and four full thickness sections from each quadrant were collected.

2.4.2 Placental reporting

All sections were stained with H&E and reported by an experienced perinatal pathologist (Dr. Margaret Evans). Placental reaction patterns were reported according to the site of inflammation, using a structured system (Redline et al. 2003). Histologic chorioamnionitis was defined as the presence of an inflammatory response in the placental membranes of any grade or stage. Maternal and fetal inflammatory responses were defined as shown in Table 2-2.

Maternal inflammatory response (MIR)	**Fetal inflammatory response (FIR)**
Chorionitis	Vasculitis
Chorioamnionitis	Funisitis
Necrotising chorioamnionitis	
Intervillositis	

Table 2-2. Definitions of maternal and fetal inflammatory responses.

2.5 MRI data collection

2.5.1 MRI procedure

Structural and diffusion MRI brain scans were performed on a Siemens Magnetom Verio 3T scanner (Siemens Healthcare Gmbh, Erlangen, Germany) using a 16-channel matrix phase array head coil. Infants were scanned at term-equivalent age (37-44 weeks) in natural sleep with monitoring of pulse oximetry, electrocardiography and temperature. For ear

protection, flexible earplugs and neonatal earmuffs (MiniMuffs, Natus Medical Inc., CA) were used. I was responsible for the medical supervision of infants during scanning with assistance from research midwife, Gillian Lamb.

2.5.2 Imaging acquisition protocol

Infants were scanned to acquire: 3D T1-weighted (T1w) MPRAGE volume (TR= 1970 ms, TE= 4.69 ms, inversion time= 1100 ms, flip angle= 9° acquisition plane= sagittal, voxel size= 1 x 1 x 1 mm^3, FOV= 160 mm, acquisition time= 3:09 min), axial T2-weighted BLADE (TR=4100 ms, TE= 207 ms, voxel size= 0.7 x 0.7 x 3.0mm^3, FOV= 220 mm, acquisition time= 2:29 min), T2-weighted SPACE (TR=3200 ms, TE= 409 ms, acquisition plane= sagittal, voxel size 1 x 1 x 1 mm^3, FOV= 128 mm, acquisition time= 2:13 min), SWI (TR= 28 ms, TE= 20 ms, voxel size= 0.8 x 0.8 x 3.0 mm^3, acquisition time= 2:23 min) and axial T2-weighted FLAIR BLADE (TR= 10000, TE= 130, voxel size= 0.9 x 0.9 x 3.0 mm^3, acquisition time= 3:22 min). Diffusion MRI data were acquired in two parts with interspersed T2-weighted (b=0 s/mm^2) volumes: part 1 consisting of 8 b=0 s/mm^2 and 64 diffusion-weighted b=750 s/mm^2, and part 2 consisting of 8 b=0 s/mm^2, 3 b=200 s/mm^2, 6 b=500 s/mm^2 and 64 b=2500 s/mm^2 single-shot spin-echo echo planar imaging (EPI) volumes acquired with 2 mm isotropic voxels (TR= 3500ms, TE= 78 ms, FOV= 256 mm, acquired matrix 128 x 128, acquisition time 4:29 + 5:01 min).

2.5.3 Structural reporting

Structural images were reported by a Paediatric Radiologist with experience in neonatal MRI (Dr. Alan J. Quigley) using an established system (Leuchter et al. 2014). Images with evidence of post-haemorrhagic ventricular dilatation, cystic periventricular leukomalacia or central nervous system malformation were excluded from subsequent analysis.

2.5.4 Pre-processing

Digital Imaging and Communication on Medicine (DICOM) image files for both structural and diffusion MRI were converted to Neuroimaging Informatics Technology Initiative (NIFTI) format. Diffusion MRI volumes were denoised using a Marchenko-Pastur-PCA based algorithm (Veraart et al. 2016; Tournier et al. 2019). Eddy current, head movement and EPI geometric distortions were corrected using outlier replacement and slice-to-volume registration (Andersson and Sotiropoulos 2016; Andersson et al. 2016; Andersson et al. 2017; Jenkinson et al. 2012; Andersson, Skare, and Ashburner 2003) and bias field inhomogeneity correction was applied (Tustison et al. 2010).

2.5.5 Peak width of skeletonised water diffusion parameters

A multi-modality template was constructed using data from 50 term born infants with DTI-TK and all the subjects were aligned (Blesa et al. 2020; Zhang et al. 2006). The water diffusion tensor derived maps (MD, AD, RD and FA) of each subject were calculated after registration. The NODDI maps were calculated in the subject's native space with the NODDI-Bingham model using cuDIMOT (Tariq et al. 2016; Hernandez-Fernandez et al. 2019) and then the intracellular volume fraction [NDI] was then propagated to the template space using the previously calculated transformations. The main FA skeleton template was created by thresholding at 0.15, and individual FA maps were projected onto this skeleton. Using this projection, the remaining water diffusivity and NODDI maps were also projected onto the white matter skeleton. A custom mask was created by editing the skeleton mask to remove CSF and grey matter contaminated areas, and by removing tracts passing through the cerebellum, the brainstem and subcortical grey matter areas using ITK-Snap (Yushkevich et al. 2006). The resulting skeletonized maps were then multiplied by the custom mask. Peak width skeletonised mean diffusivity (PSMD) and neurite density index (PSNDI) were calculated as the difference between the 95th and 5th percentiles on histogram analysis (Blesa et al. 2020). An overview of the pipeline is shown in Figure 2-1. Pre-

processing and generation of peak width skeletonised water diffusion parameters was performed by Dr. Manuel Blesa.

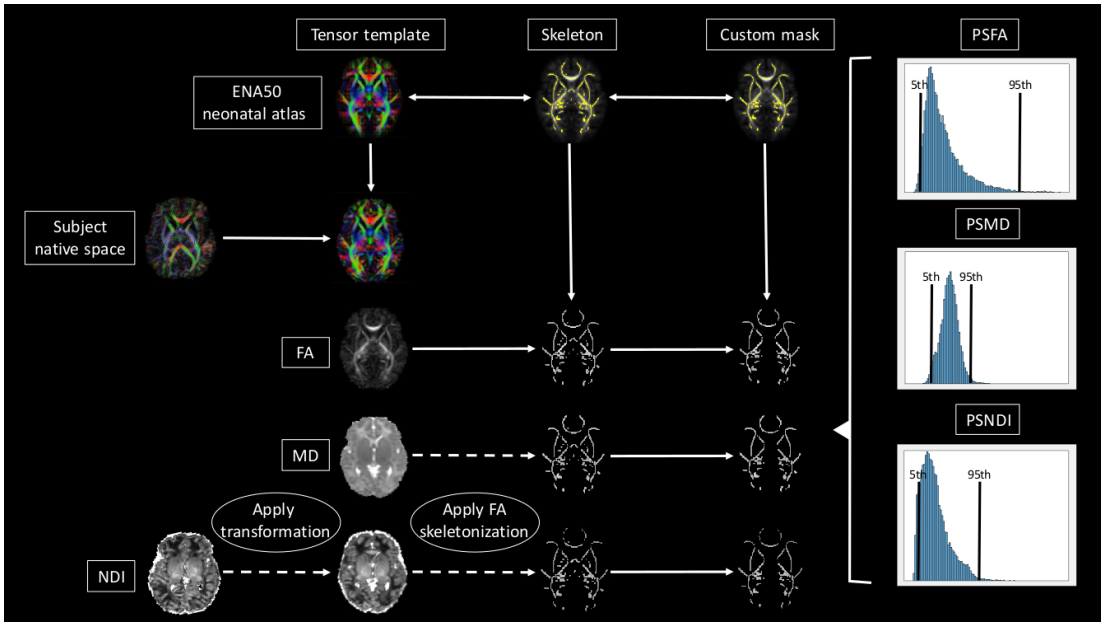

Figure 2-1. Pipeline for the calculation of peak width skeletonised water diffusion parameters.
Reproduced with permission (Blesa et al. 2020).

2.6 Generation of iPSC-derived cortical neurons

2.6.1 Maintenance of iPSCs

All iPSCs were derived from human donor skin fibroblasts using an episomal non-integrating approach (Okita et al. 2011) under full ethical and IRB approval of the University of Edinburgh. The healthy control iPSC line used in this study has been previously described (Vasistha et al. 2019).

iPSCs were maintained in 1:60 Matrigel (BD Biosciences) coated plastic dishes in E8 medium (Life Technologies) at 37°C in 5% CO_2. Media was changed daily, and cells were passaged using a Dispase/Collagenase enzyme mix (1:1) once they reached 90% confluence.

2.6.2 Generation of anterior NPCs

iPSCs were differentiated into anterior NPCs (aNPCs) by Dr. Karen Burr

using an established neural conversion protocol (Bilican et al. 2014; Chambers et al. 2009; Stacpoole et al. 2011). Dual SMAD inhibition (blocking two signalling pathways used by SMADs: bone morphogenic protein, BMP and transforming growth factor β, TGF-β) was used to differentiate iPSCs into neuroepithelial rosettes, which assume a default obligate anterior identity in chemically defined medium (CDM). Following 7 days in neuralisation medium, this was changed to base media (A-DMEM/F12, 1% P/S, 1% Glutamax, 1% N2), 0.4% B27, 2.5ng/mL FGF2. On day 18-22, neural rosettes were mechanically isolated, dissociated using Accutase (Sigma) and plated as a monolayer in plates coated with 1:100 Laminin and suspended in proliferation media (Base media, 0.1% B27, 10ng/mL FGF2). Media was changed every 48 hours and aNPCs were grown to 100% confluence.

2.6.3 Differentiation of aNPCs into cortical neuronal cultures

24-well plates with glass coverslips were prepared with a Poly-D-ornithine (Sigma) overnight incubation followed by 3 washes with cell culture grade water, 1-hour UV and then coated with 1:10 diluted Matrigel (BD Biosciences), 20 µg/ml Fibronectin (Sigma) and 10 µg/ml Laminin (Sigma). aNPCs were plated in default medium (A-DMEM/F12, 1% P/S, 0.5% Glutamax, 0.5% N2, 0.2% B27 and 2 µg/ml Heparin) at a density of 250,000 per well and incubated at 37°C in 3% Oxygen. On day 7, default media was supplemented with 10 µM forskolin (Tocris) to encourage cell cycle exit. Cells were fed twice weekly throughout the protocol.

2.6.4 GFP labelling of aNPCs

aNPCs were transduced with lentivirus expressing GFP at a multiplicity of infection (MOI:1) which allowed sparse labelling before platedown. The generation of GFP lentivrus was performed by Dr. Pamela Brown (Biomolecular Core, University of Edinburgh Shared University Research Facility) and the transduction of aNPCs was performed by Dr. Bhuvaneish Selvaraj.

2.6.5 Karyotyping

Standard G banding chromosome analysis was performed in The Doctors Lab (TDL), London, UK, to confirm chromosome number and genetic abnormality throughout the course of this study.

2.7 Immunohistochemistry

2.7.1 Staining protocol

All steps were performed at room temperature. Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes and then washed three times with PBS. Cells were permeabilised with 0.1% Triton X-100 and then blocked with 6% goat serum for 30 minutes. Samples were then incubated with primary antibodies in 6% goat serum for 1 hour followed by Alexa Fluor-labelled secondary antibodies in 6% goat serum for 1 hour. Nuclei were counter-stained with DAPI (Sigma) (5µg/ml). After final washes in PBS, coverslips were mounted on glass slides using Fluorsave™ reagent.

The proportion of mitotic cells was studied by labelling with 10µM ethynyl deoxyuridine (EdU) for 24 hours. Media was replaced completely the following day and cells cultured for an additional 48 hours in the absence of EdU before fixation using 4% PFA. For detection, cells were permeabilised using 0.5% Triton X-100 and labelled using the EdU click-iT detection kit (Thermo-Fisher) according to manufacturer's instructions. Details of antibodies used are shown in Table 2-3.

Primary antibody	Isotype	Manufacturer	Concentration	Secondary antibody
OTX2	Goat	R&D Systems	1:100	A11055 Donkey anti-goat 488
PAX6	Rb	BioLegend	1:100	A11008 Goat anti-rabbit 488
TBR2	Rb	Abcam	1:100	A11008 Goat anti-rabbit 488
Nestin	Mouse IgG1	Millipore	1:200	A21121 Goat anti-mouse IgG1 488
TUBB3	Mouse IgG2b	Sigma	1:1000	A21242 Goat anti-mouse IgG2b 647
GFAP	Rb	Dako	1:1000	A21428 Goat anti-rabbit 555

Table 2-3. Primary and secondary antibodies.

2.7.2 Imaging

Images were taken on a Zeiss Observer Z1 wide field microscope. Images were acquired with identical settings for parallel cultures. Regions of interest were identified by uniform nuclear staining and 3 regions were imaged per coverslip.

Representative images were prepared with ImageJ (NIH). Images were then converted to TIFF format and analysed using Definiens Developer in collaboration with Dr. James Longden, to identify cell counts, EdU quantification and neurite length.

3 The inflammatory profile after preterm birth

3.1 Introduction

Histologic chorioamnionitis (HCA) is a leading cause of preterm birth (Romero et al. 2001; Goldenberg, Hauth, and Andrews 2000) and affects 40-70% of very preterm infants (Dammann et al. 2004). Acute inflammation is characterised by the infiltration of neutrophils first into the choriodecidual junction and then into the fetal tissues. Histologically defined maternal inflammatory response (MIR) refers to infiltration of the fetal chorion and amnion (chorioamnionitis), whilst fetal inflammatory response (FIR) describes involvement of the blood vessels of the chorionic plate (vasculitis) or the umbilical cord (funisitis) (Cappelletti, Presicce, and Kallapur 2020). HCA is complicated by FIR in approximately 30% of very preterm infants with increasing risk associated with lower gestational age (Hecht et al. 2008; Mestan et al. 2009).

HCA is associated with an increased risk of neonatal mortality and morbidity including lung disease, IVH, sepsis and necrotising enterocolitis (Bose et al. 2011; Villamor-Martinez, Álvarez-Fuente, et al. 2019; Been et al. 2013; Hofer et al. 2013; Ozalkaya et al. 2016; Francis et al. 2019). HCA has also been implicated in the development of white matter injury, CP and neurodevelopmental impairment (Venkatesh et al. 2020; Shatrov et al. 2010; Leviton et al. 2010; Anblagan et al. 2016). When HCA involves both MIR and FIR, these risks appear to be increased further, suggesting that organ injury is mediated by a systemic fetal inflammatory response syndrome (FIRS). FIRS was initially defined as IL-6 >11ng/mL in umbilical cord blood (Gomez et al. 1998; Gotsch et al. 2007) but subsequent studies have shown an association between placental FIR and elevations in several circulating factors that reflect endothelial activation, leukocyte migration and the acute phase response (Hecht et al. 2011; Døllner et al. 2002; Mestan et al. 2009; Armstrong-Wells et al. 2015) leaving uncertainty about which immune mediator(s) best predict FIRS.

Preterm infants have a distinct immune profile in umbilical cord blood when compared to term-born controls but this can be influenced by antenatal factors, environmental exposures and developmental regulation (Matoba et al. 2009). HCA has been associated with elevated cytokines (IL-1β, IL-6 and TNF-α), chemokines (IL-8, MCP-1, MIP-1β, RANTES), matrix metalloproteinases (MMP-1 and MMP-9) and CRP in umbilical cord blood (Hecht et al. 2011; Døllner et al. 2002; Mestan et al. 2009; Armstrong-Wells et al. 2015; Kacerovsky et al. 2013). Blood concentrations of inflammation-related proteins are also more likely to remain elevated postnatally in preterm infants who were exposed to HCA (Leviton, Hecht, et al. 2011). If the period of immune dysregulation triggered by HCA is sustained, this suggests that mediators of the inflammatory response may provide a link between HCA and organ damage and that there may be a therapeutic window of opportunity for intervention following delivery. Given the delay before placental histopathology can be performed after birth, there remains an unmet need to identify a robust blood-based biomarker of FIRS to identify infants at highest risk of organ damage who may benefit from targeted anti-inflammatory or immunomodulatory therapies.

In this study, an immunoassay of 24 analytes customised to reflect the perinatal innate and adaptive immune response was used to analyse profiles from umbilical cord and postnatal blood with placental histopathology to test the hypotheses that (1) preterm birth is associated with a distinct pro-inflammatory profile when compared to term-born controls, (2) specific inflammatory mediators in umbilical cord blood are associated with histologic chorioamnionitis and the fetal inflammatory response, and (3) exposure to histologic chorioamnionitis is associated with an altered immune profile on day 5 after preterm birth.

3.2 Methods

3.2.1 Participants

Participants were 192 infants, 133 preterm infants born $\leq 32^{+0}$ weeks' gestation and 59 healthy term-born controls born $>37^{+0}$ weeks, delivered at the Royal Infirmary of Edinburgh, UK and recruited to the Theirworld Edinburgh Birth Cohort Study.

3.2.2 Dried blood spot collection

Dried blood spot samples (DBSS) were taken from the umbilical cord following delivery for both preterm cases and term-born controls. For preterm infants, an additional sample was collected on day 5 of life. A customized multiple sandwich immunoassay based on meso-scale technology was used to measure blood spot levels of Interleukin(IL)1-β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, Monocyte chemotactic protein-1 (MCP-1), Macrophage inflammatory protein-1α (MIP-1α), Macrophage inflammatory protein-1β (MIP-1β), Tumor necrosis factor-α (TNF-α), Tumor necrosis factor-β (TNF-β), Brain-derived neurotropic factor (BDNF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon-γ (IFN-γ), C-reactive protein (CRP), matrix-metalloproteinase 9 (MMP-9), Regulated upon activation, normal T cell expressed and secreted (RANTES) and Complement components C3, C5a and C9.

DBSS has been shown to be a robust method of handling samples for immunoassay analysis of inflammatory markers in whole blood with (Skogstrand et al. 2005). For a detailed description of the DBSS collection procedure, processing and analysis see Chapter 2, section 2.3.

3.2.3 Placental histopathology

Placental examination was performed by an experienced perinatal pathologist (M.J.E.) and placental reaction patterns were reported according

to the site of inflammation, using a structured system (Redline et al. 2003). Histologic chorioamnionitis (HCA) was defined as the presence of an inflammatory response in the placental membranes of any grade or stage. Maternal inflammatory response (MIR) was defined as the presence of chorionitis, chorioamnionitis or intervillositis. Fetal inflammatory response (FIR) was defined as the presence of vasculitis in the chorionic plate or funisitis involving any vessel of the umbilical cord.

3.2.4 Statistical analysis

Participant characteristics were compared using Student's T test or Mann-Whitney U to compare distributions, and Chi-square tests were used to compare proportions. Analytes with values less than the level of detection (<LOD) were assigned the lowest detectable level prior to statistical analysis, and analytes with concentrations <LOD in ≥75% of samples were excluded from subsequent statistical analysis.

To investigate group differences in cord blood immune mediator profiles between preterm infants and term-born controls, the Mann-Whitney U was used with Bonferroni correction for multiple tests. A principal component analysis (PCA) was used to identify analytes contributing to variance in the cord blood profile and analytes that contributed to PCs with eigenvalues >1 were then entered as independent variables in a logistic regression model to predict preterm or term category. Analytes contributing to variability within PCs predictive of gestational category were then investigated individually using Pearson correlation to identify developmentally regulated analytes most strongly correlated with gestational age.

To investigate group differences in immune mediator profiles in preterm infants between those with and without exposure to HCA the Mann Whitney-U was used with Bonferroni correction for multiple tests. Associations that remained significant after correction were analysed in logistic regression models where HCA was the dependent variable, cord blood analyte

concentration was the independent variable, and GA at birth was entered as a covariate. The predictive power of those analytes that were associated with HCA after adjustment for GA at birth was determined using receiver operator characteristic (ROC) analysis. Exploratory analyses were performed to compare the immune mediator profiles in umbilical cord blood and postnatal day 5 blood for infants exposed to HCA with placental MIR+FIR- versus MIR+FIR+ using Mann Whitney U. Statistical analyses were performed using SPSS version 24.0 (IBM Corp., Armonk, NY), with the exception of PCA, which was performed using R version 3.6.1 (R Core Team, 2019).

3.3 Results

3.3.1 Participant demographics

DBSS were obtained for 133 preterm infants and 59 term-born controls. Clinical characteristics are shown in Table 3-1.

Not all participants had data collected at all timepoints so subsets were used to test individual hypotheses.

	Preterm n= 133	**Term n= 59**
Mean gestational age, weeks (SD)	29^{+0} (2.24) range 23^{+2} to 32^{+0}	39^{+4} (1.04) range 37^{+3} to 42^{+0}
Birthweight, g (SD)	1223 (406) range 454-2110	3549 (515) range 2556-4800
Male sex, n (%)	76 (57)	30 (51)
Delivery mode, n (%): Vaginal Emergency caesarean Elective caesarean	 53 (40) 80 (60)	 23 (39) 10 (17) 26 (44)
SIMD, n (%) 1 2 3 4 5 missing	 23 (17) 27 (20) 28 (21) 22 (17) 29 (22) 4 (3)	 5 (8) 6 (10) 11 (19) 17 (29) 20 (34)
Histologic chorioamnionitis, n (%) MIR+ FIR- MIR+ FIR+	42 (31) 19 (14) 23 (17)	6 (10) 1 (2) 5 (8)
Antenatal steroids, n (%)	127 (96)	
Magnesium sulphate, n (%)	122 (92)	
Prolonged ROM, n (%)	29 (22)	
Early onset sepsis	9/124 (7)	
Late onset sepsis	16/121 (13)	
Bronchopulmonary dysplasia	34/121 (26)	
Necrotising enterocolitis Medical management Surgery	9/120 (8) 3/120 (3) 6/120 (5)	
Retinopathy of prematurity	8/118 (6)	
Deaths before discharge	10/133 (8)	

Table 3-1. Clinical characteristics of participants.

3.3.2 Umbilical cord blood profile associated with prematurity

DBSS were obtained from the umbilical cord of 55 preterm infants and 59 term born controls. 10 analytes (GM-CSF, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17, MIP-1α and TNF-β) were <LOD in ≥75% of samples and were therefore excluded from subsequent analysis. Following Bonferroni correction

significant group differences remained for 9 immune mediators (p<0.004). Median and IQR of analytes are shown in Table 3-2.

	Preterm n=55		**Term n=59**		
Analyte (pg/ml)	**Median**	**Q1,Q3**	**Median**	**Q1,Q3**	**P value**
BDNF	22.27	6.04, 36.90	62.33	43.18, 113.68	<0.001
C3	3081996	1858061, 4569906	4447459	3520958, 5613383	<0.001
C5a	3694	2421, 11471	7136	4060, 9444	0.028
C9	1237	215, 6902	12629	3609, 43156	<0.001
CRP	100.88	89.00, 12054.79	89.00	89.00, 89.00	<0.001
IL-1β	0.26	0.26, 0.49	0.11	0.02, 0.34	0.009
IL-6	0.45	0.45, 6.00	0.45	0.45, 0.45	<0.001
IL-8	12.05	5.75, 81.08	8.29	4.89, 14.71	0.049
IL-18	25.62	10.35, 38.34	41.27	27.50, 54.78	<0.001
MCP-1	109.09	68.69, 210.75	48.12	38.54, 66.63	<0.001
MIP-1β	12.01	7.47, 18.82	9.72	6.88, 18.24	0.397
MMP-9	14001	4486, 40077	155886	87195, 358085	<0.001
RANTES	1460	703, 2754	3128	1817, 5295	<0.001
TNF-α	0.27	0.27, 0.37	0.27	0.27, 0.27	0.521

Table 3-2. Cord blood analytes.

Pro-inflammatory proteins IL-6, MCP-1 and CRP were elevated in the cord blood of preterm infants whilst BDNF, C3, C9, IL-18, MMP-9 and RANTES were increased in term-born controls.

PCA showed that the first five principal components (eigenvalues>1) explained 76% of the variance in the cord blood profile (Table 3-3), with the majority of variance explained by the first two (25% and 20% respectively). Projection of individual inflammatory profiles onto the first two principal components is shown in Figure 3-1.

Component	Eigenvalue	Total variance /%	Cumulative variance /%
1	3.56	25.43	25.43
2	2.85	20.33	45.76
3	1.88	13.44	59.20
4	1.29	9.21	68.42
5	1.10	7.84	76.25
6	0.85	6.07	82.32
7	0.58	4.13	86.45
8	0.50	3.55	90.00
9	0.46	3.32	93.31
10	0.31	2.22	95.53
11	0.20	1.45	96.98
12	0.19	1.38	98.36
13	0.18	1.31	99.67
14	0.05	0.33	100.00

Table 3-3. Variance in the cord blood inflammatory profile.

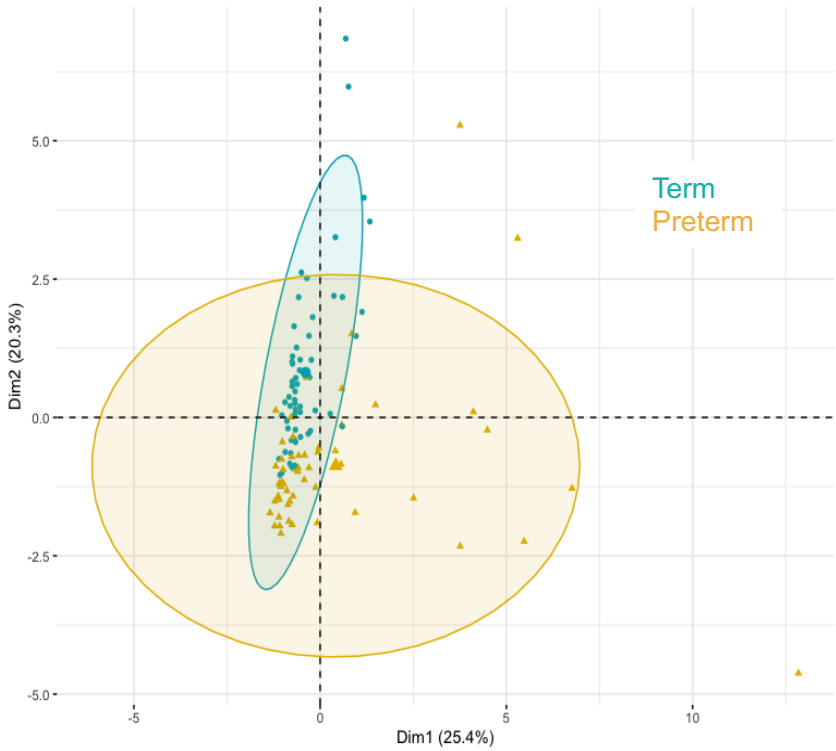

Figure 3-1. Projection of individual cord blood inflammatory profiles onto the first two principal components, grouped by gestational age category.

In a logistic regression model to predict preterm or term category, principal components 1, 2 and 5 were found to be predictive of gestational age with a classification accuracy of 86% (95% CI 0.78-0.92), p value= 1.242e-14. The contribution of individual components to the regression model are shown in Table 3-4 and the percentage contribution of each analyte to variability in the cord blood profile is shown in Figure 3-2.

	B	**SE B**	**β**	**p value**
PC1	2.8189	0.7863	5.3424	0.000337
PC2	-2.6162	0.5467	-4.4331	1.71e-06
PC3	-0.1343	0.2946	-0.1850	0.648519
PC4	0.8840	0.5917	1.0084	0.135172
PC5	-0.8628	0.3907	-0.9076	0.027207

Table 3-4. Logistic regression for the prediction of gestational age category using principal components derived from the umbilical cord blood profile.

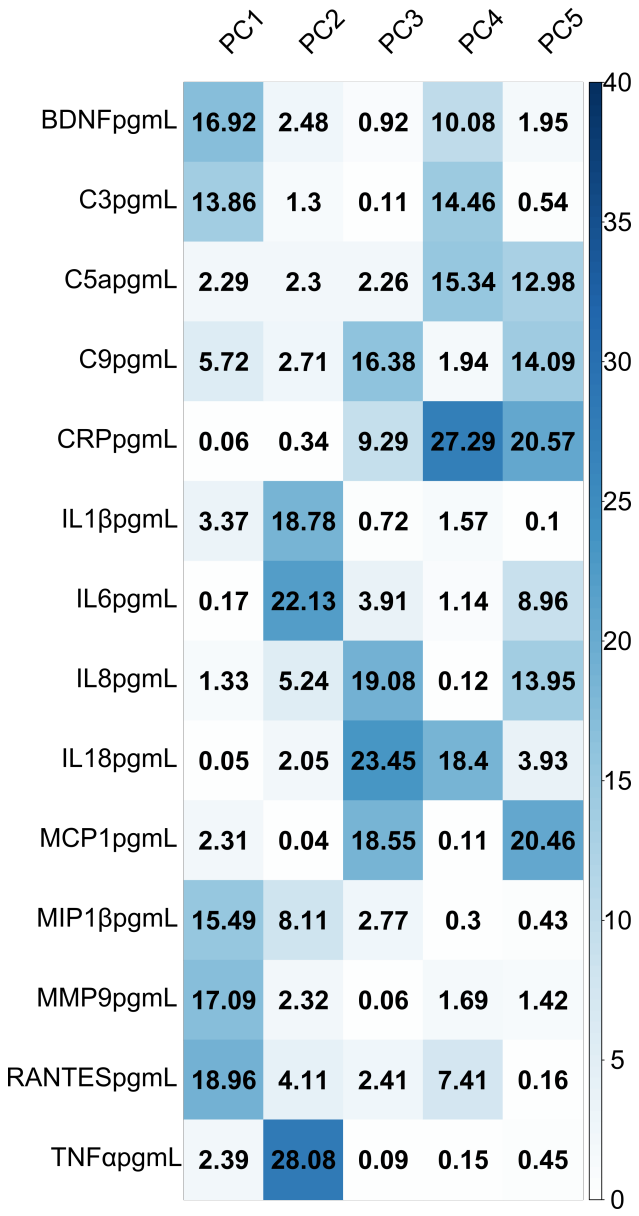

Figure 3-2. The percentage contribution of each analyte to variability in the cord blood profile.

Amongst immune mediators contributing to variability within the principal components most predictive of gestational category, correlation analysis showed that cord blood BDNF was the most highly correlated with gestational age at birth (r= 0.555, p= 1.5433e-10). Correlation coefficients for individual analytes are shown in Table 3-5.

Analyte	Correlation coefficient	p value
BDNF	0.555	1.5433e-10
MMP-9	0.482	5.6601e-8
RANTES	0.311	0.001
C3	0.229	0.014
MIP-1β	-0.100	0.290
IL-1β	-0.105	0.266
TNF-α	0.024	0.799

Table 3-5. Correlation between individual analytes and gestational age.

3.3.3 Umbilical cord blood mediators associated with histologic chorioamnionitis

Of 55 infants with umbilical cord blood sampling and placental histopathology, 24 (44%) were exposed to HCA during fetal life. Infants with HCA exposure had lower GA at birth than infants without HCA: mean GA 28^{+0} weeks versus 29^{+5} weeks (p= 0.002), and were more likely to have prolonged rupture of membranes (ROM) prior to delivery (p=0.007). There were no statistically significant group differences in birthweight, infant sex, delivery mode or exposures to antenatal corticosteroids or magnesium sulphate for fetal neuroprotection. Participant characteristics are shown in Table 3-6 and umbilical cord blood analyte concentrations are shown in Table 3-7.

	No HCA n= 31	HCA n= 24
Gestational age, weeks (SD)	29^{+5} (1.69)	28^{+0} (2.75)
Birthweight, g (SD)	1236 (420)	1157 (476)
Male sex, n (%)	21 (68)	12 (50)
Delivery mode, n (%): Vaginal Caesarean	 11 (36) 20 (64)	 12 (50) 12 (50)
Antenatal steroids, n (%)	31 (100)	23 (96)
Magnesium sulphate, n (%)	29 (94)	23 (96)
Prolonged ROM, n (%)	4 (13)	11 (46)

Table 3-6. Characteristics of infants exposed to HCA compared to those not exposed.

	No HCA n=31		HCA n=24		
Analyte (pg/ml)	**Median**	**Q1,Q3**	**Median**	**Q1,Q3**	**p value**
BDNF	21.40	4.15, 30.85	29.33	6.24, 48.22	0.072
C3	2451737	1655564, 3373401	3859628	2265099, 6336222	0.03
C5a	2780	1943, 4172	6202	3612, 16933	<0.001
C9	583	153, 1483	4857	1285, 38747	<0.001
CRP	89.00	89.00,195.02	8829.84	89.00, 100702.67	0.001
IL-1β	0.03	0.03, 0.03	0.49	0.03, 0.93	<0.001
IL-6	0.45	0.45, 0.45	5.20	0.56, 23.18	<0.001
IL-8	7.54	3.38, 12.06	106.70	25.54, 496.42	<0.001
IL-18	28.06	15.38, 47.92	14.49	8.39, 35.76	0.06
MCP-1	85.40	65.06, 117.17	206.84	104.07, 364.51	<0.001
MIP-1β	8.91	4.32, 16.84	16.13	11.07, 29.87	0.04
MMP-9	11681	2139, 20169	16664	5242, 70314	0.127
RANTES	1311	618, 2754	1541	716, 3350	0.519
TNF-α	0.27	0.27, 0.56	0.27	0.27, 0.35	0.619

Table 3-7. Umbilical cord blood analyte concentrations for infants exposed to HCA compared to those not exposed.

Those exposed to HCA had higher cord blood levels of C5a, C9, CRP, IL-1β, IL-6, IL-8 and MCP-1 ($p<0.003$, Bonferroni corrected). Of these 7 analytes, 5 were associated with HCA after adjustment for GA at birth in regression models: C5a, IL-1β, IL-6, IL-8 and MCP-1 (β between 0.88 and 28.78, $p< 0.05$). IL-8 concentration was the best predictor of HCA with an area under

the curve (AUC) of 0.917 (SE 0.039), 95% CI 0.841 – 0.993, p<0.001. Table 3-8 shows the AUC for each analyte and Figure 3-3 shows the ROC analysis.

Analyte (pg/mL)	AUC (SE)	95% CI	p value
IL-8	0.917 (.039)	0.841- 0.993	1.434e-07
IL-6	0.848 (.056)	0.739- 0.958	0.000011
IL-1β	0.845 (.059)	0.729- 0.961	0.000013
MCP-1	0.786 (.066)	0.657- 0.916	0.000301
C5a	0.778 (.064)	0.651- 0.904	0.000457
C9	0.776 (.066)	0.646- 0.905	0.001
CRP	0.754 (.071)	0.614- 0.894	0.001

Table 3-8. Area under the curve for prediction of HCA using cord blood analytes.

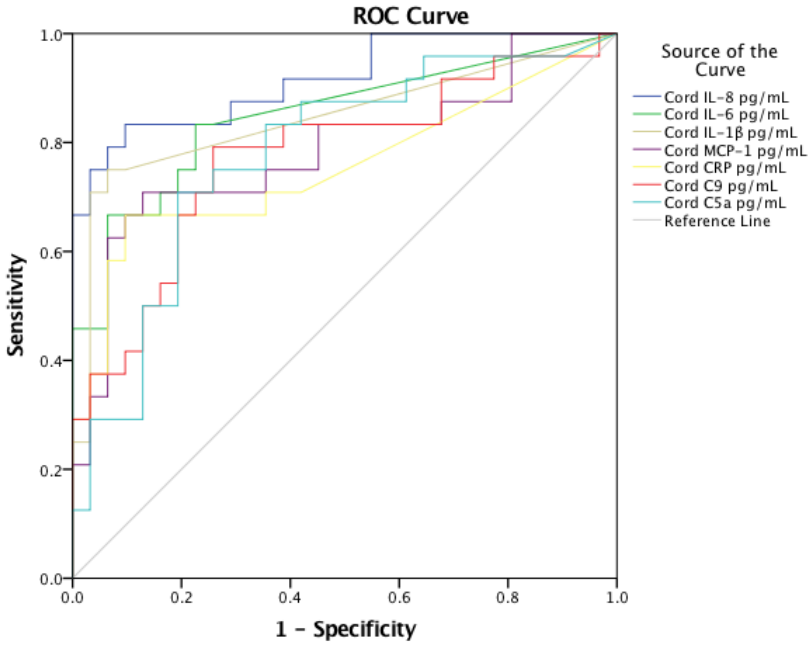

Figure 3-3. ROC curve analysis of cord blood analytes for the prediction of HCA.

In an exploratory analysis of the 24 infants with HCA, 13 (54%) had evidence of FIR (MIR+FIR+). Of the 14 analytes investigated, only concentrations of IL-1β, IL-6 and IL-8 in umbilical cord blood were associated with exposure to FIR (p<0.004, Bonferroni corrected), Figure 3-4.

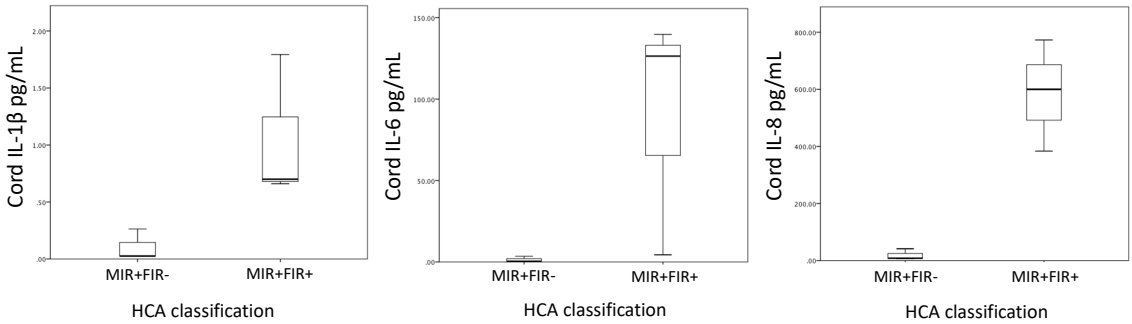

Figure 3-4. Boxplots of cord blood analytes associated with evidence of placental FIR.

3.3.4 Histologic chorioamnionitis is associated with an altered immune profile on day 5 after preterm birth

Of 98 preterm infants with day 5 DBSS, 31 (32%) were exposed to HCA during fetal life. Infants with HCA exposure had lower GA at birth than infants without HCA: mean GA 28^{+2} weeks versus 29^{+4} weeks (p=0.004). Infants exposed to HCA were more likely to have been delivered vaginally (p<0.001) and more likely to have prolonged ROM prior to delivery (p<0.001). There were no statistically significant group differences in birthweight, infant sex or exposures to antenatal corticosteroids or magnesium sulphate. Participant characteristics are shown in Table 3-9.

	No HCA n= 67	**HCA n= 31**
Gestational age, weeks (SD)	29^{+4} (1.84)	28^{+2} (2.63)
Birthweight, g (SD)	1248 (368)	1187 (421)
Male sex, n (%)	39 (58)	18 (58)
Delivery mode, n (%): Vaginal Caesarean	 16 (24) 51 (76)	 23 (74) 8 (26)
Antenatal steroids, n (%)	63 (94)	30 (97)
Magnesium sulphate, n (%)	61 (91)	30 (97)
Prolonged ROM, n (%)	8 (11)	14 (45)
EOS, n (%)	4 (6)	4 (13)

Table 3-9. Characteristics of infants with day 5 DBSS who were exposed to HCA compared to those not exposed.

5 immune proteins on day 5 of life had a median level in preterm infants exposed to HCA outside the IQR for preterm infants who were not exposed, highlighted in bold (Table 3-10). Day 5 concentrations of C3 and MMP-9 remained significantly associated with HCA after Bonferroni correction ($p<0.004$).

	No HCA n=67		**HCA n=31**		
Analyte (pg/ml)	**Median**	**Q1,Q3**	**Median**	**Q1,Q3**	**p value**
BDNF	**28.49**	**14.36, 42.11**	**42.28**	**26.16, 100.09**	**0.008**
C3	**3584454**	**2761051, 4909173**	**5084307**	**4180180, 6912830**	**<0.001**
C5a	6556	4236, 10086	9505	7337, 13451	0.002
C9	7463	2526, 20740	18110	4761, 55877	0.028
CRP	251.05	89.00, 8496.84	1431.89	89.00, 25859.42	0.194
IL-1β	0.03	0.03, 0.08	0.05	0.03, 0.11	0.065
IL-6	0.45	0.45, .045	0.45	0.45, 0.45	0.641
IL-8	**11.44**	**7.56, 19.44**	**29.26**	**12.55, 39.53**	**0.012**
IL-18	35.78	22.68, 53.77	34.35	21.98, 47.92	0.501
MCP-1	136.03	108.84, 203.28	106.97	90.22, 148.65	0.010
MIP-1β	**7.88**	**5.50, 11.12**	**12.04**	**7.22, 16.22**	**0.011**
MMP-9	**56654**	**28342, 98231**	**240084**	**64199, 460437**	**<0.001**
RANTES	2606.92	929.93, 4196.25	3486.66	2058.30, 5362.46	0.070
TNF-α	0.27	0.27, 0.27	0.27	0.27, 0.27	0.986

Table 3-10. Day 5 blood analyte concentrations for infants exposed to HCA compared to those not exposed.
Analytes with a median level in preterm infants exposed to HCA outside the IQR for preterm infants without HCA are highlighted in bold.

In an exploratory analysis of the 31 infants with HCA, 18 (58%) had evidence of FIR (MIR+FIR+). Of the 14 analytes investigated, only C3 concentration in postnatal day 5 blood was found to be associated with exposure to FIR ($p<0.004$, Bonferroni corrected), Figure 3-5.

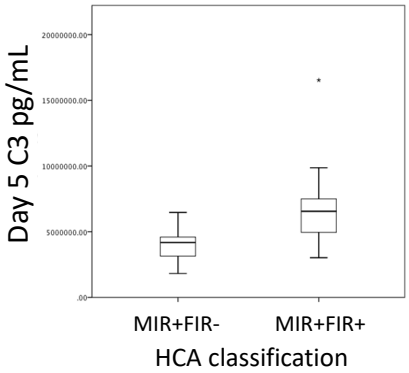

Figure 3-5. Boxplot of day 5 C3 in infants with evidence of placental FIR.

33 infants had blood obtained from the umbilical cord at delivery and on postnatal day 5. Of 14 (42%) with HCA, 8 (57%) had evidence of FIR (MIR+FIR+). Longitudinal analysis confirmed that IL-1β, IL-6 and IL-8 were elevated in the umbilical cord blood of infants with FIR but were less discriminatory by postnatal day 5, whilst C3 remained significantly altered. Line graphs demonstrating analyte trajectories from birth to postnatal day 5 are shown in Figure 3-6.

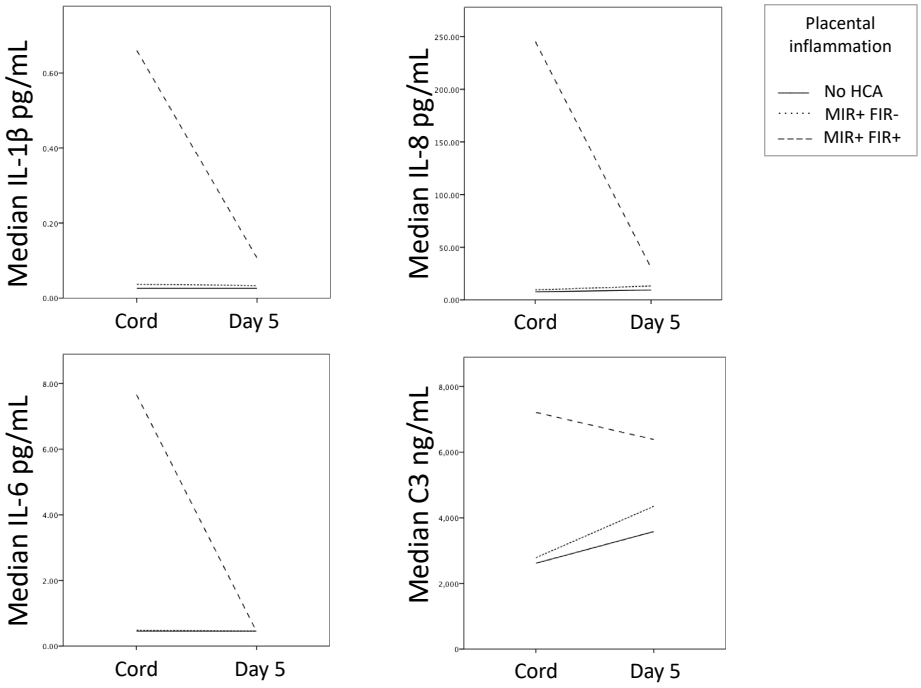

Figure 3-6. Analyte trajectories from umbilical cord to postnatal day 5.

3.4 Discussion

By combining placental histopathology with a customised array of immune mediators in umbilical cord blood and postnatal blood from a large group of mother-infant dyads, this study characterises marked differences in the systemic immune profile of preterm infants compared with term controls, highlights specific mediators that are highly predictive of HCA, and demonstrates that in preterm infants the immune profile of those exposed to HCA remains altered on day 5 after birth.

The umbilical cord blood immune profile was distinctly pro-inflammatory in preterm infants with significant elevations in 3 markers of the acute phase response: IL-6, MCP-1 and CRP. In contrast, 6 proteins were elevated in term-born controls, suggesting developmental regulation: BDNF C3, C9, IL-18, MMP-9 and RANTES. BDNF was the analyte most highly correlated with gestational age. BDNF belongs to the family of neurotrophins: an important group of signalling molecules responsible for neuronal growth, maturation

and synaptic plasticity during development (Huang and Reichardt 2001). Prematurity, placental dysfunction and fetal growth restriction have all been associated with reduced levels of BDNF (Matoba et al. 2011; Leviton et al. 2017; D'Angelo et al. 2020; Flöck et al. 2016) which may have important implications for long-term brain health. Reduced BDNF in the neonatal period has been associated with increased risk of developing ASD (Skogstrand et al. 2019) whilst elevated BDNF in the weeks after preterm birth is associated with better cognitive performance in childhood (Kuban et al. 2018). IL-18, MMP-9 and RANTES are known to be influenced by both gestational and postnatal age (Leviton, Fichorova, et al. 2011; Królak-Olejnik, Beck, and Olejnik 2006; Flöck et al. 2016) and the complement system is under-developed in preterm infants (McGreal, Hearne, and Spiller 2012; Grumach et al. 2014).

This study demonstrates a strong association between histologic markers of placental inflammation and a systemic inflammatory response in the newborn. Umbilical cord blood IL-8 concentration was found to be the best predictor of exposure to HCA and exploratory analyses suggest that this relationship is driven by placental FIR. Several studies have shown that IL-8 synthesis is enhanced in neonates (Schultz et al. 2002; Strunk et al. 2004; Thornton, Cody, and Yost 2012) and predictive of inflammatory complications such as sepsis and necrotising enterocolitis (Leviton et al. 2012; Zhou et al. 2015; Wong et al. 2008; Maheshwari et al. 2014; Benkoe et al. 2014; Satar et al. 2008), suggesting that IL-8 is a promising biomarker of the neonatal systemic inflammatory response.

This study also suggests that the systemic inflammatory response to histologic chorioamnionitis is sustained beyond delivery in preterm infants. Previous studies have demonstrated that the neonatal inflammatory response can be dysregulated and prolonged in the weeks after preterm birth, particularly following funisitis, which may be due to the intensity of the inflammatory response, impaired resolution, developmental regulation or

even genetic risk (Skogstrand et al. 2008; Leviton, Hecht, et al. 2011; Liston, Carr, and Linterman 2016). In this study, C3 and MMP-9 were elevated on day 5 after preterm birth in infants exposed to HCA in utero. Exploratory analyses suggest that placental FIR results in an altered trajectory of C3 expression but contribution of adverse postnatal events to the day 5 immune profile cannot be excluded.

The complement cascade plays a key role in the innate immune response (Merle, Noe, et al. 2015) but is a potent inflammatory system which when dysregulated can cause significant tissue damage following injury. The complement cascade can be activated through several mechanisms but all component pathways converge at Complement protein C3 (Merle, Church, et al. 2015). C3 participates in multiple key processes affecting developing brain architecture, including tagging of synapses for pruning by microglia (Schafer et al. 2012; Gorelik et al. 2018). Regulators of complement are low in preterm infants (Grumach et al. 2014) and this may contribute to an uncontrolled complement response in the context of neuroinflammation (Fragopoulou et al. 2019) and brain injury secondary to hypoxia-ischaemia (Rocha-Ferreira and Hristova 2015). Numerous studies beyond the neonatal period have also implicated complement C3 dysregulation in CNS pathology including neurodevelopmental disorders (Magdalon et al. 2020), multiple sclerosis (Werneburg et al. 2020), traumatic brain injury (Alawieh et al. 2018) and neurodegeneration (Wu et al. 2019).

MMP-9 is a member of the zinc-dependent endopeptidases that prototypically cleave extracellular matrix (ECM), cell adhesion molecules and cell surface receptors. Matrix-metalloproteinases also modulate the inflammatory response through the regulation of endothelial barrier function, cytokine activity and chemotactic gradient formation but the outcome of proteolysis is context specific (Fingleton 2017).
The ECM is a key regulator of neural network development and plasticity through the stabilisation of synaptic contacts. Dysregulation of MMP-9 during

a critical window of CNS vulnerability may therefore have long-term consequences on structural connectivity (Reinhard, Razak, and Ethell 2015). MMP-9 is higher in the CSF of preterm infants with post haemorrhagic ventricular dilatation (PHVD) when compared to those without brain injury (Okamoto et al. 2010) and elevated peripheral MMP-9 is associated hypoxia-ischaemia in animal models, correlating with severity of injury in human infants born at term (Walsh et al. 2011; Bednarek et al. 2012; Savard et al. 2015).

Whilst this study analysed a large number of inflammation-associated proteins that are important in the innate and adaptive immune response in the newborn, a limitation is that the cytokine response is governed not only by environmental insults but also by genetic factors and maternal inflammatory status (Dammann and O'Shea 2008; Holst and Garnier 2008; Sheikh et al. 2016). Potential risks or resilience conferred by the genome or maternal immune influences were not able to be examined. Instead antenatal inflammation was characterised using placental histopathology, which is most closely associated with fetal inflammatory responses.

Exploratory longitudinal analysis suggests that the systemic immune response to HCA is sustained but cross-sectional data analysis on day 5 may be confounded by neonatal complications such as invasive ventilation, sepsis and IVH.

The rate of placental FIR was high in this study (54-58% of all HCA cases were MIR+FIR+) but sample size did not permit sub-group analysis based on gestational age. Given the developmental regulation of immune responses and the increased risk of FIR with lower gestational age, this would be interesting to consider in future work.

Studies have shown that analytes measured from DBSS correlate with plasma concentrations, but it is recognised that these methods are not

interchangeable (Massaro et al. 2019). In this study, no analyte concentrations were above the upper limit of detection but several participants had analyte values below the lower limits of detection. The majority of term-born controls had CRP and TNF-α concentrations <LOD whilst values for IL-4 and IL-10 were <LOD in all participants. Whilst these results may reflect the reduced incidence of systemic inflammation in healthy term infants and low circulating levels of anti-inflammatory proteins in all newborns, future studies are needed to explore the balance of damaging and protective factors.

Whilst efforts were made to facilitate the recruitment of infants from a variety of sources, the demographics of families who were approached to participate but did not give consent were not able to be examined and so potential sampling bias could not be explored. However, participants were successfully recruited from all five SIMD quintiles and the proportion of infants delivered vaginally was similar in both groups.

3.5 Conclusions

By combining placental histopathology with a comprehensive assessment of immune mediators, this study shows that preterm infants have a distinct pro-inflammatory profile in umbilical cord blood at birth and IL-8 concentration predicts exposure to HCA. The immune profile of infants exposed to HCA remains altered on day 5 after birth, suggesting that there may be a therapeutic window for targeted intervention using anti-inflammatory or immunomodulatory therapies to reduce the risk of complications associated with activation of the fetal inflammatory response.

4 IL-8 is implicated in brain dysmaturation following preterm birth

The work presented in this chapter is published in:

Sullivan, G., Galdi, P., Cabez, M.B., Borbye-Lorenzen, N., Stoye, D.Q., Lamb, G.J., Evans, M.J., Quigley, A.J., Thrippleton, M.J., Skogstrand, K., Chandran, S., Bastin, M.E., Boardman, J.P., 2020. Interleukin-8 dysregulation is implicated in brain dysmaturation following preterm birth. Brain, behavior, and immunity 90, 311-318.

4.1 Introduction

Preterm birth affects around 15 million births annually (Chawanpaiboon et al. 2019), and is an important cause of CP, cognitive impairment, autism spectrum disorder and psychiatric disease later in life (Twilhaar et al. 2018). MRI studies reveal a cerebral phenotype of preterm birth that is associated with later function, and includes diffuse white matter disease, dysconnectivity of developing networks, and structural alterations in cortical and deep grey matter (Boardman et al. 2010; Kapellou et al. 2006; Ball et al. 2017; Galdi et al. 2020; Batalle et al. 2017). These image features are manifest by term-equivalent age, which suggests that interventions to prevent injury and restore typical development may need to be applied during the perinatal period.

Immune dysregulation at a critical point in neurodevelopment is strongly implicated in the pathogenesis of the encephalopathy of prematurity, which can be conceptualised as white matter injury and subsequent dysmaturation of diverse cellular processes resulting in atypical development of white and grey matter structures (Volpe 2019; Hagberg et al. 2015). Preterm infants have a distinct inflammatory profile in blood and CSF that includes higher

levels of pro-inflammatory cytokines and lower levels of neuroprotective growth factors compared to infants born at term (Skogstrand et al. 2008; Boardman et al. 2018), and there is evidence that both systemic inflammation and neuroinflammation are associated with overt forms of preterm brain injury. For example, elevated IL-1β, IL-6, IL-8 and IL-10 in umbilical cord and early postnatal blood is associated with intraventricular hemorrhage and white matter lesions soon after birth (Yoon et al. 1996; Duggan et al. 2001; Ellison et al. 2005; Leviton et al. 2018), and persistently elevated pro-inflammatory proteins during the first 2 weeks after preterm birth are associated with increased risk of cerebral palsy (Kuban et al. 2014; Carlo et al. 2011) and impaired neurocognitive development in infancy and childhood (O'Shea et al. 2012; O'Shea et al. 2013; Hansen-Pupp et al. 2008; Kuban et al. 2014; Leviton et al. 2016; Yanni et al. 2017; Kuban et al. 2017). Furthermore, specific co-morbidities of preterm birth characterised by systemic inflammation, including histologic chorioamnionitis and necrotising enterocolitis, are associated with abnormal white matter on MRI (Shah et al. 2008; Ball et al. 2017; Anblagan et al. 2016; Barnett et al. 2018). However, previous study designs leave uncertainty about which immune mediators are associated with white matter dysmaturation.

Diffusion tensor imaging (DTI) and neurite orientation and dispersion imaging (NODDI) support inference about the microstructural properties and geometrical organisation of developing white matter (Zhang, Schneider, et al. 2012; Tariq et al. 2016; Pietsch et al. 2019). The integration of DTI scalars and NODDI metrics have recently been optimised for studying the neonatal brain and histogram analyses provide a single measure of generalised white matter microstructure. Peak width of skeletonised mean diffusivity (PSMD) and peak width of skeletonised neurite density index (PSNDI) are both altered in preterm infants at term-equivalent age when compared to term-born controls, indicating that these are useful biomarkers of white matter connectivity in the developing brain (Blesa et al. 2020).

Identification of immune mediators of generalised dysconnectivity during the perinatal period is important for elucidating new targets for neuroprotection therapies. In this study, an immunoassay of 24 analytes customised to reflect perinatal innate and adaptive immune responses, was used to analyse postnatal day 5 blood samples with brain MRI to test the hypothesis that specific immune mediators link systemic inflammation with atypical white matter development in preterm infants.

4.2 Materials and methods

4.2.1 Participants

Participants were 71 preterm infants born before $\leq 32^{+0}$ weeks' gestation, delivered at the Royal Infirmary of Edinburgh, UK and recruited to the Theirworld Edinburgh Birth Cohort Study. Exclusion criteria included infants with major congenital abnormality, post-haemorrhagic ventricular dilatation defined as ventricular indices >97th centile +4mm, cystic periventricular leukomalacia, or contra-indications to 3T MRI.

4.2.2 Blood sample collection

Dried blood spot samples (DBSS) were taken from the umbilical cord following delivery and from blood drawn on day 5 of life using a FTA™ DMPK-A Card (Whatman™ GE Healthcare). Bloodspot cards were dried at room temperature and then stored at -20°C until analysis at the Statens Serum Institut (Center for Neonatal Screening, Copenhagen, Denmark). A customised multiple sandwich immunoassay based on meso-scale technology was used to measure blood spot levels of Interleukin(IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, Monocyte chemotactic protein-1 (MCP-1), Macrophage inflammatory protein-1α (MIP-1α), Macrophage inflammatory protein-1β (MIP-1β), Tumor necrosis factor-α (TNF-α), Tumor necrosis factor-β (TNF-β), Brain-derived neurotropic factor (BDNF), Granulocyte-macrophage colony-stimulating factor (GM-CSF),

Interferon-γ (IFN-γ), C-reactive protein (CRP), matrix-metalloproteinase 9 (MMP-9), Regulated upon activation, normal T cell expressed and secreted (RANTES) and Complement components C3, C5a and C9. A detailed description of the DBSS collection procedure, processing and analysis are provided in Chapter 2, section 2.3.

4.2.3 MRI analysis

Figure 4-1 summarises the pipeline for calculation of histogram-based metrics. A detailed description of MRI procedure, sequence acquisition and image processing are provided in Chapter 2, section 2.5.

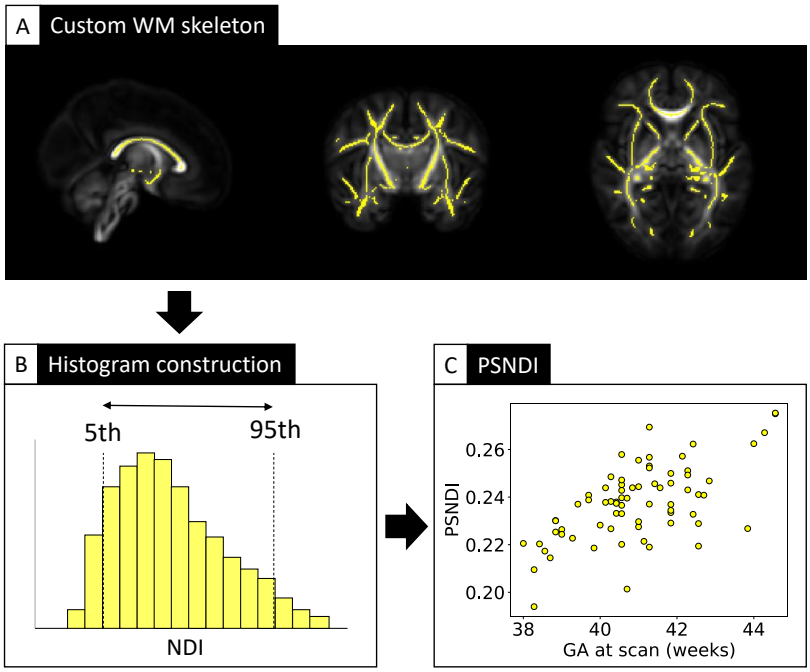

Figure 4-1. Imaging pipeline for the construction of PSNDI.
(A) shows the custom white matter skeleton, (B) shows the calculation of PSNDI from a histogram analysis of neurite density index, and (C) shows a scatterplot of PSNDI versus gestational age at MRI scan.

4.2.4 Statistical analysis

Participant characteristics were compared using Student's t-test or Mann-Whitney U to compare distributions, and Chi-square tests were used to compare proportions. Analytes with values less than the lower limit of

detection (<LOD) were assigned the lowest detectable level prior to statistical analysis, and analytes with concentrations <LOD in ≥75% of participants were excluded.

To investigate relationships between systemic inflammation and white matter microstructure, principal component analysis was used to identify blood analytes contributing to variance in the postnatal day 5 inflammatory profile. Analytes that contributed to principal components with eigenvalues >1 were entered as independent variables in multivariable linear regression models with PSMD and PSNDI as dependent variables, and GA at birth and scan as covariates.

Statistical analyses were performed using SPSS version 24.0 (IBM Corp., Armonk, NY), with the exception of PCA, which was performed using R version 3.6.1 (R Core Team, 2019).

4.3 Results

4.3.1 Participants

71 preterm infants (delivered ≤ 32^{+0} weeks of gestation) had blood taken on postnatal day 5 and MRI performed at term-equivalent age (Figure 4-2). Clinical characteristics of participants are shown in Table 4-1.

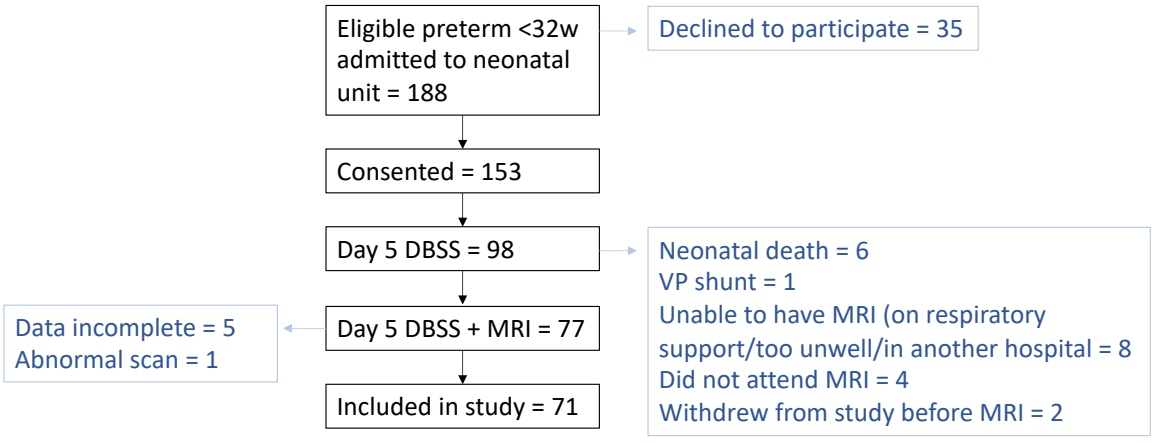

Figure 4-2. Recruitment flowchart.

Mean GA at birth, weeks (range)	29^{+4} (23^{+6} - 32^{+0})
Mean birthweight, g (range)	1284 (600-2060)
Proportion of male infants (%)	58
Antenatal steroids, any (%)	96
Antenatal magnesium sulphate, any (%)	93
Histologic chorioamnionitis, HCA (%)	30
Sepsis (%)	18
Necrotising enterocolitis, NEC (%)	6
Bronchopulmonary dysplasia, BPD (%)	25
Retinopathy of prematurity, ROP (%)	6
Mean GA at scan, weeks (range)	41^{+0} (38^{+0}-44^{+4})

Table 4-1. Clinical characteristics of participants.

4.3.2 Postnatal inflammation and white matter microstructure

The mean PSNDI for the group was 0.238 mm^2sec^{-1} $x10^{-3}$ (SD 0.016), and mean PSMD was 0.601 mm^2sec^{-1} $x10^{-3}$ (SD 0.062).

Using PCA, we found that five principal components (eigenvalues >1) explained 66% of the variance in the postnatal inflammatory profile, with most variance explained by the first two (23% and 15% respectively). Cumulative variance explained by all components is shown in Table 4-2.

Component	Eigenvalue	Total variance /%	Cumulative variance /%
1	3.17	22.62	22.62
2	2.14	15.28	37.89
3	1.69	12.08	49.97
4	1.27	9.06	59.02
5	1.03	7.38	66.40
6	0.97	6.94	73.35
7	0.83	5.91	79.26
8	0.66	4.69	83.94
9	0.57	4.10	88.05
10	0.54	3.82	91.87
11	0.39	2.77	94.64
12	0.38	2.71	97.35
13	0.24	1.69	99.04
14	0.13	0.96	100.00

Table 4-2. Variance in the postnatal day 5 inflammatory profile.

Fourteen analytes contributed to the first 5 principal components: BDNF, C3, C5a, C9, CRP, IL-1β, IL-6, IL-8, IL-18, MCP-1, MIP-1β, MMP-9, RANTES and TNF-α. Individual contributions are shown in Figure 4-3.

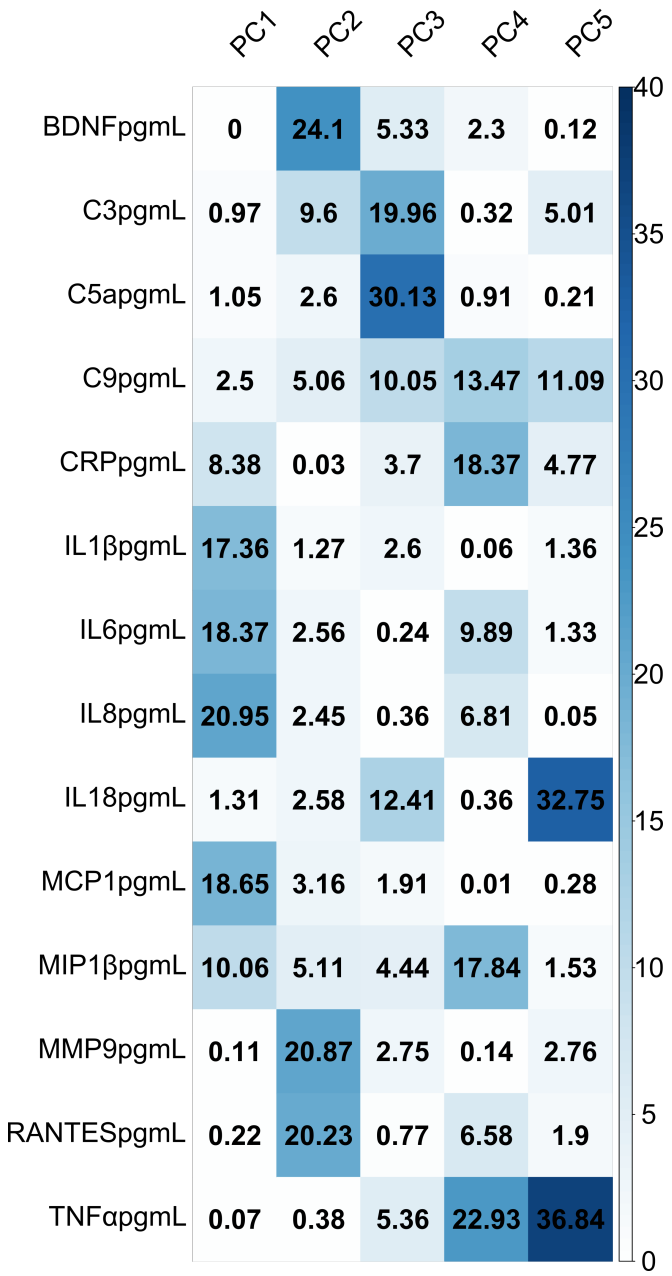

Figure 4-3. The percentage contribution of each analyte to variability in the day 5 blood profile.

Of these 14 analytes, IL-8 was the only one to be associated with PSNDI at term equivalent age (β=0.221, p=0.037), in a multivariable model that explained 48% of the variance in PSNDI at term-equivalent age (F (16,54)= 5.09, R^2 adjusted=0.48, p<0.001), Table 4-3. Partial regression of day 5 IL-8 and PSNDI is shown in Figure 4-4.

	B	SE B	β
Constant	-1.574e-01	5.168e-02	
IL-8	1.258e-04	5.874e-05	.221
GA	3.066e-03	9.007e-04	.369
PMA at scan	7.370e-03	1.084e-03	.706

Table 4-3. Multivariable regression for the prediction of PSNDI using day 5 analytes.

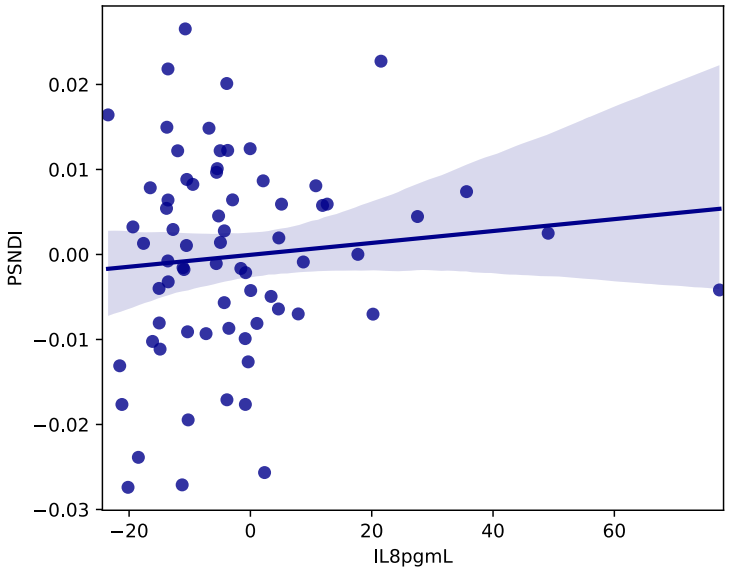

Figure 4-4. Partial regression plot of day 5 IL-8 and PSNDI.

None of the remaining 13 analytes were associated with PSNDI and there were no significant associations between any analyte and PSMD.

4.4 Discussion

By combining data from the postnatal immune profile with brain diffusion MRI, this study shows that elevated IL-8 in the first week of life is associated with white matter dysmaturation at term-equivalent age.

IL-8 is a member of the CXC chemokine family and a mediator of the systemic inflammatory response, where its key role is neutrophil chemotaxis.

IL-8 can be secreted by innate and adaptive immune cells and a variety of CNS cells including astrocytes and microglia (Choi et al. 2014; Rustenhoven et al. 2016). IL-8 binds to target cells via the G-protein coupled receptors CXCR1 and CXCR2. These receptors are expressed throughout the CNS and are associated with key neurodevelopmental processes including cell migration, proliferation and differentiation, oligodendrocyte maturation, axonal growth and synaptic plasticity (Ubogu, Cossoy, and Ransohoff 2006; Semple, Kossmann, and Morganti-Kossmann 2010; Watson et al. 2020; Deverman and Patterson 2009). This suggests multiple potential mechanisms through which IL-8 dysregulation during a critical window of neurodevelopment may disrupt healthy CNS development.

IL-8 dysregulation has been implicated in the pathology of several types of human brain injury across the life course. It is associated with blood brain barrier dysfunction in adult traumatic brain injury (Kossmann et al. 1997; Obermeier, Daneman, and Ransohoff 2013), with altered cerebral metabolism and poor neurodevelopmental outcome following neonatal hypoxic ischaemic injury (Bartha et al. 2004; Foster-Barber, Dickens, and Ferriero 2001) and with increased mortality in children following traumatic brain injury (Woodcock and Morganti-Kossmann 2013). Studies focused on the perinatal period report that elevated IL-8 in blood sampled from the umbilical cord or soon after birth is associated with overt white matter injury and cerebral palsy, neurodevelopmental impairment, and cognitive impairment in children born preterm, and it is one of the neonatal cytokines that is most strongly associated with subsequent diagnosis of autism among children born at term (Kuban et al. 2017; Leviton et al. 2019; Carlo et al. 2011; Kuban et al. 2014; Silveira and Procianoy 2011; Heuer et al. 2019; Kinjo et al. 2011; Hansen-Pupp et al. 2008). Furthermore, studies investigating the maternal immune activation hypothesis and offspring mental health report that elevated maternal IL-8 during pregnancy is associated with alterations in brain structure and an increased risk of schizophrenia in offspring (Ellman et al. 2010; Brown et al. 2004). This accumulating evidence

suggests that inflammation associated with elevated IL-8 has a significant adverse impact on the developing brain with pervasive effects on both structure and function.

To the author's knowledge, this is the first study to integrate mediators of systemic inflammation with biomarkers of white matter microstructure in a representative group of preterm infants without major parenchymal brain injury. Strengths of the study include the selection of a large number of inflammation-associated proteins that are important in the innate and adaptive immune response in the newborn, and use of a data driven approach based on PCA to characterise the inflammatory profile associated with white matter microstructure. The choice of image features was principled, based on established characterisations of white matter dysmaturation in preterm infants, including water content and dendritic/axonal complexity within the white matter skeleton (Blesa et al. 2020; Kunz et al. 2014b; Lynch et al. 2020).

A limitation of the study is that potential risks or resilience conferred by the genome could not be explored and the sample size was not sufficient to perform sub-group analyses based on gestational age.
Global measures of white matter microstructure were selected as microstructural properties have been shown to be substantially shared across the major white matter tracts of the newborn brain (Telford et al. 2017), and it was hypothesised that systemic inflammation exerts a global effect; different methods, such as those based on quantitative tractography, would be required to investigate hypotheses about tract specific or regional susceptibilities to immune dysregulation.

4.5 Conclusions

This study provides further support for a substantial role of generalised inflammation in the aetiology of preterm brain injury, and suggests that IL-8

dysregulation may provide a link between systemic inflammation and brain dysmaturation. Given the potential role of immunomodulatory therapies for treating diseases of the central nervous system (Çakici et al. 2019; Wittenberg et al. 2019), and the availability of anti-IL-8 monoclonal antibody therapies, this finding has implications for the development of perinatal neuroprotection strategies based on anti-inflammatory and novel immune therapeutics.

5 The effect of inflammation on the development of iPSC-derived cortical neurons

5.1 Introduction

Preterm birth is associated with an increased risk of neurocognitive problems involving executive function, attention, language processing, working memory and emotional regulation. Grey matter injury is a feature of the encephalopathy of prematurity and cortical dysmaturation may contribute to the cognitive and behavioural disturbances observed in preterm infants (Fleiss, Gressens, and Stolp 2020; Volpe 2019).

MRI studies have shown that preterm infants have reduced volume of the cerebral cortex (Inder et al. 2005; Makropoulos et al. 2016), altered cortical microstructural organisation (Ball, Srinivasan, et al. 2013; Bouyssi-Kobar et al. 2018) and dysconnectivity of developing neural networks (Ball, Boardman, et al. 2013) when compared to term-born controls at term-equivalent age. These cortical alterations persist into childhood (Zhang et al. 2015; Peterson et al. 2000) and adolescence (Nosarti et al. 2008) and are associated with neurocognitive outcomes (Kapellou et al. 2006; Keunen et al. 2016; Bora et al. 2014; Rathbone et al. 2011; Gui et al. 2019; Ball et al. 2015; Kline et al. 2019).

However, the neurobiology of these imaging signatures remains unknown. The cerebral cortex is a complex and highly organised 6-layered structure composed predominantly of projection neurons which are glutamatergic and extend long range axons to other cortical regions and subcortical structures, and inhibitory gamma-aminobutyric acid (GABA)-expressing interneurons which usually project shorter axons within the same cortical region (Franco and Müller 2013). Both glutamatergic neurons and interneurons are affected by preterm birth (Malik et al. 2013; Stolp et al. 2019). Understanding the molecular and cellular mechanisms responsible for abnormal cortical

development is a crucial next step towards the development of neuroprotective strategies for preterm infants.

All neurons and glia of the CNS are derived from neuroepithelial cells of the developing brain (Stiles and Jernigan 2010). Neural progenitor cells in the proliferative ventricular zone (VZ) are mitotic and initially undergo symmetrical divisions to increase the precursor pool whilst the neural plate is forming. Following closure of the neural tube on gestational week (GW) 4, neural progenitors transition into radial glia (RG) cells with potential to generate both neurons and glia. RG express PAX-6 and divide asymmetrically to create a proliferating RG and an immature neuron or a RG and an intermediate progenitor cell (IPC) (Taverna, Gotz, and Huttner 2014). IPCs can divide symmetrically to amplify the precursor pool further or undergo terminal division to create post-mitotic excitatory glutamatergic neurons. Once formed, neurons migrate radially from the proliferative regions of the VZ in an organised way to form the developing six layered neocortex with the deepest layer populated first, shown in Figure 5-1 (Molnár et al. 2019).

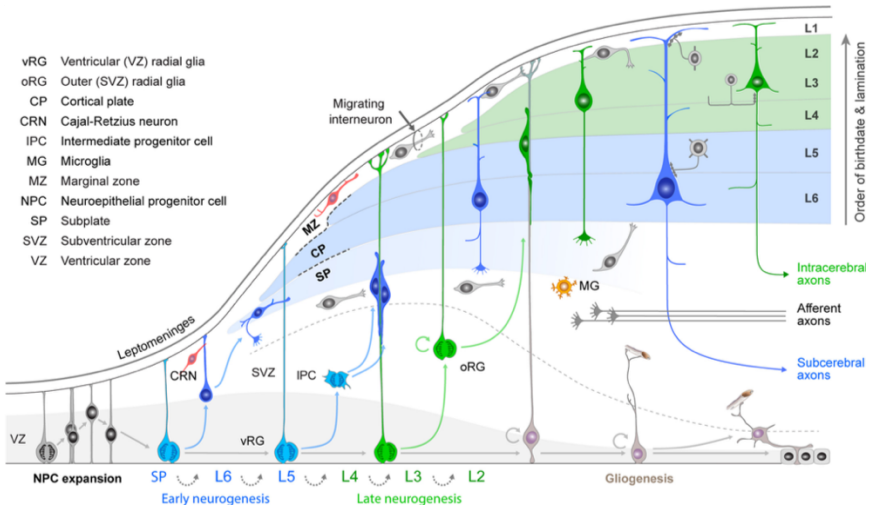

Figure 5-1. Schematic illustration of mammalian neurogenesis.
Reproduced with permission from (Molnár 2019), Copyright John Wiley & Sons, Inc.

By PCW 24, the cerebral cortex has been populated by the full complement of excitatory projection neurons, whilst tangential migration of interneurons

from the dorsal and ventral telencephalon occurs later, reaching peak density at term (Xu et al. 2011; Arshad et al. 2016). During migration, immature neurons extend and retract neurites which later become axons and dendrites once polarisation is determined.

Cortical development during the third trimester of pregnancy represents a period of rapid brain growth with many complex dynamic cellular processes vulnerable to injury (Andescavage et al. 2017; Makropoulos et al. 2016). Highly regulated spatiotemporal gene expression is crucial for the cellular composition and cytoarchitecture of the developing neocortex (Kang et al. 2011; Nowakowski et al. 2017) and the timing of progenitor cell expansion is a key determinant of cortical size (Picco et al. 2018). Single cell RNA sequencing of the human prefrontal cortex showed that RG in PCW 9 to 16 were actively proliferating with a switch to neuronal differentiation in PCW 16 to 26. This is accompanied by neural network formation, with genes related to axonal generation expressed in PCW 19 to 26 and synaptogenesis from PCW 23 (Zhong et al. 2018).

Cytokines and chemokines are implicated in several key pathways for healthy cortical development, including neural progenitor cell proliferation, migration and fate determination (Deverman and Patterson 2009; Guidolin, Fede, and Tortorella 2019; Mousa and Bakhiet 2013). Perturbation of these processes due to inflammatory exposures in utero may contribute to the alterations in neural circuitry observed in association with preterm birth. For example, maternal immune activation has been associated with reduced NPC proliferation and premature cell cycle exit with resulting alterations in neuronal subtypes in the fetal cortex of offspring (Carpentier et al. 2013; Stolp et al. 2011; Stojanovska et al. 2018; Hester et al. 2018; Bilbo et al. 2018). Whilst mechanistic links remain speculative, evidence from animal models suggests that dysregulation of cytokine networks can lead to significant alterations in fetal brain development (Gregg and Weiss 2005; Ma et al. 2014; Bernardino et al. 2008; Ahn, Lee, and Kim 2015; Neumann et al. 2002; Veerasammy et al. 2020) and chemokines have been implicated in the

regulation of progenitor cell pluripotency (Jiang et al. 2017; Jung et al. 2015; Krtolica et al. 2011).

Whilst animal models offer insights into the impact of inflammation on brain structure and function, species differences in the timing of key brain maturation events can make comparisons of cellular, temporal and spatial vulnerability difficult to interpret (Semple et al. 2013; Workman et al. 2013). The developing human brain has an enhanced proliferative capacity and diverse subtypes of neural stem cells and intermediate progenitors to facilitate brain expansion, especially of the cortex (Silbereis et al. 2016). Induced pluripotent stem cells (iPSCs) reprogrammed from human somatic cells offer the opportunity to study cortical development in vitro. Functional and transcriptomic studies have shown that iPSC-derived cortical neurons have a phenotype and expression profile resembling fetal and early postnatal neurons (Stein et al. 2014; Livesey et al. 2016), making this system a promising tool to study the impact of inflammation on cortical development. Here we model the effect of immune mediators characteristic of the neonatal systemic inflammatory response (C5a, IL-6, IL-8, and TNF-α) on cortical neurogenesis and neurite outgrowth using an iPSC-derived cortical neuronal system.

5.2 Methods

5.2.1 Generation of iPSC-derived cortical neurons

All iPSCs were derived from human donor skin fibroblasts using an episomal non-integrating approach (Okita et al. 2011) under full ethical and IRB approval of the University of Edinburgh. iPSCs were converted to anterior NPCs and then excitatory glutamatergic cortical neurons using an established protocol detailed in Chapter 2, section 2.6.

5.2.2 Model characterisation

Anterior forebrain identity of NPCs was confirmed by the presence of Orthodenticle homeobox2 (OTX2). Radial glia (RG) cells were identified by the presence of Paired box 6 (PAX6), and intermediate progenitor (IP) cells by the presence of T-box protein 2 (TBR2) (Götz, Stoykova, and Gruss 1998; Englund et al. 2005).

To observe the transitional morphology of NPCs developing into cortical neurons, a time course experiment was performed from day 0 to day 14. During the experimental window from day 14 to 21, cell populations were studied to identify the representative proportion of NPCs (Nestin +), cortical neurons (ß-III tubulin +) and glial progenitors (GFAP +).

5.2.3 Experimental design

The protocol for iPSC- derived cortical neuron generation was adapted to study the effect of complement and cytokine exposure on a mixed culture of NPCs and developing cortical neurons. NPCs were plated down on day 0. On day 14, C5a, IL-6, IL-8 or TNF-α (Peprotech) was added to the media. Previous studies have shown that preterm infants may be exposed to plasma interleukin concentrations of up to 4000 pg/mL and TNF-α of 534 pg/mL (Mestan et al. 2009; Leviton, Fichorova, et al. 2011; Nielsen et al. 2015; Sullivan et al. 2020). CSF expression of C5a ranges from 420 to 5240 pg/ml and CSF IL-8 concentrations may be 10-fold higher than in plasma (Ellison 2005, Pataky 2015). Therefore, doses representative of the human inflammatory response were selected: 0.1ng/mL, 1ng/mL and 10ng/mL with an additional dose of 100ng/ml for experiments with IL-8. Cells were maintained in continuous culture with media changes every 48 hours until they were fixed for immunohistochemistry on day 21.

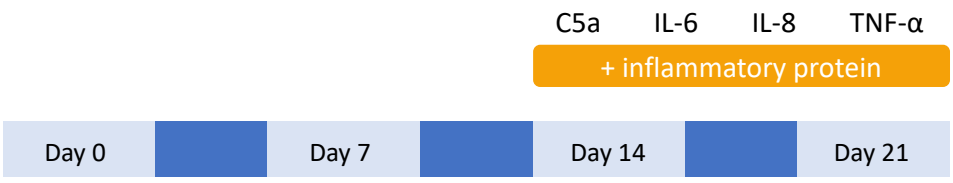

Figure 5-2. Experimental design.

Primary outcome measures were: (1) Proportion of Nestin+ NPCs, (2) Proportion of βIII tubulin+ neurons, (3) Proportion of GFAP+ astrocytes and (4) Neurite length. For experiments to measure neurite length, aNPCs were transduced with lentivirus expressing GFP at a multiplicity of infection (MOI:1) which allowed labelling before platedown. Neurite length was then calculated by measuring the maximum neurite outgrowths of GFP-labelled neurons.

5.2.4 Immunohistochemistry

Cells were fixed for immunohistochemistry on day 21. Full details of the staining protocol and antibodies used are described in Chapter 2, section 2.7. Images were taken on a Zeiss Observer Z1 wide field microscope and representative images were prepared with ImageJ (NIH). Images were then converted to TIFF format and analysed using Definiens Developer to identify cell counts, EdU quantification and neurite length.

5.2.5 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0 (GraphPad software San Diego, CA, USA). Each derivation of cortical neurons from NPCs was considered as n=1 and data was collected from a minimum of 3 derivations for each experiment (range= 3-7). Data are presented as mean +/- s.e.m. with fold change in experimental conditions compared to control. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Dunnett's test for multiple comparisons.

5.3 Results

5.3.1 *In vitro* human stem cell model to study cortical neuron development

Anterior forebrain NPCs were generated and maintained according to an established protocol (Bilican et al. 2014). Anterior forebrain identity was confirmed by the presence of Orthodenticle homeobox2 (OTX2) in cultures fixed 24 hours after plate down. In cultures fixed 14 days after platedown, RG

cells were identified by the presence of PAX6, and IP cells by the presence of TBR2 (Götz, Stoykova, and Gruss 1998; Englund et al. 2005). Images of model characterisation are shown in Figure 5-3.

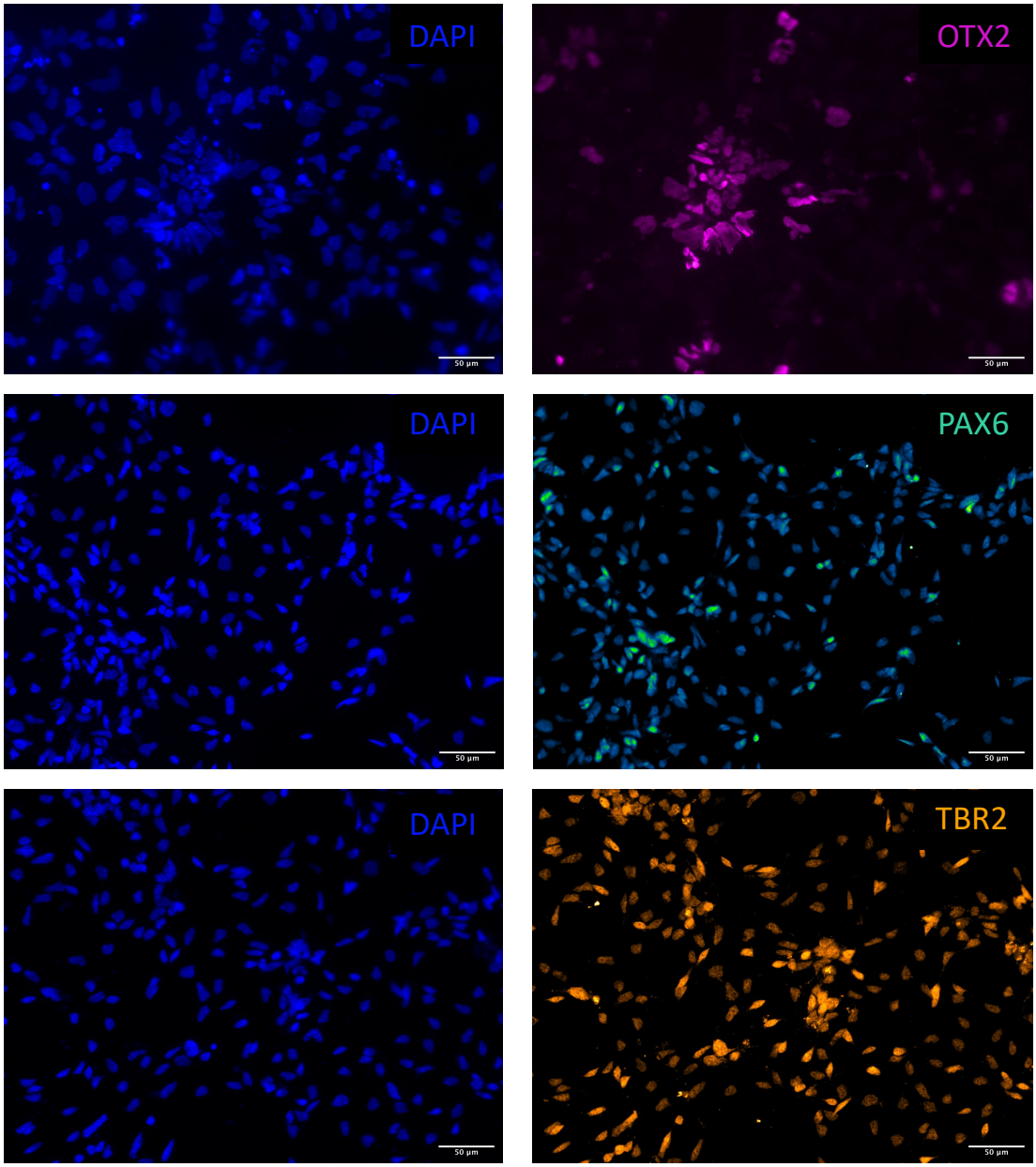

Figure 5-3. Immunostaining of model characterisation.
Representative images showing OTX2, PAX6 and TBR2 (scale bar: 50µm).

By day 14, cultures included both NPCs and developing cortical neurons. Figure 5-4 shows the transitional morphology of NPCs into neurons shown from day 0 to day 14.

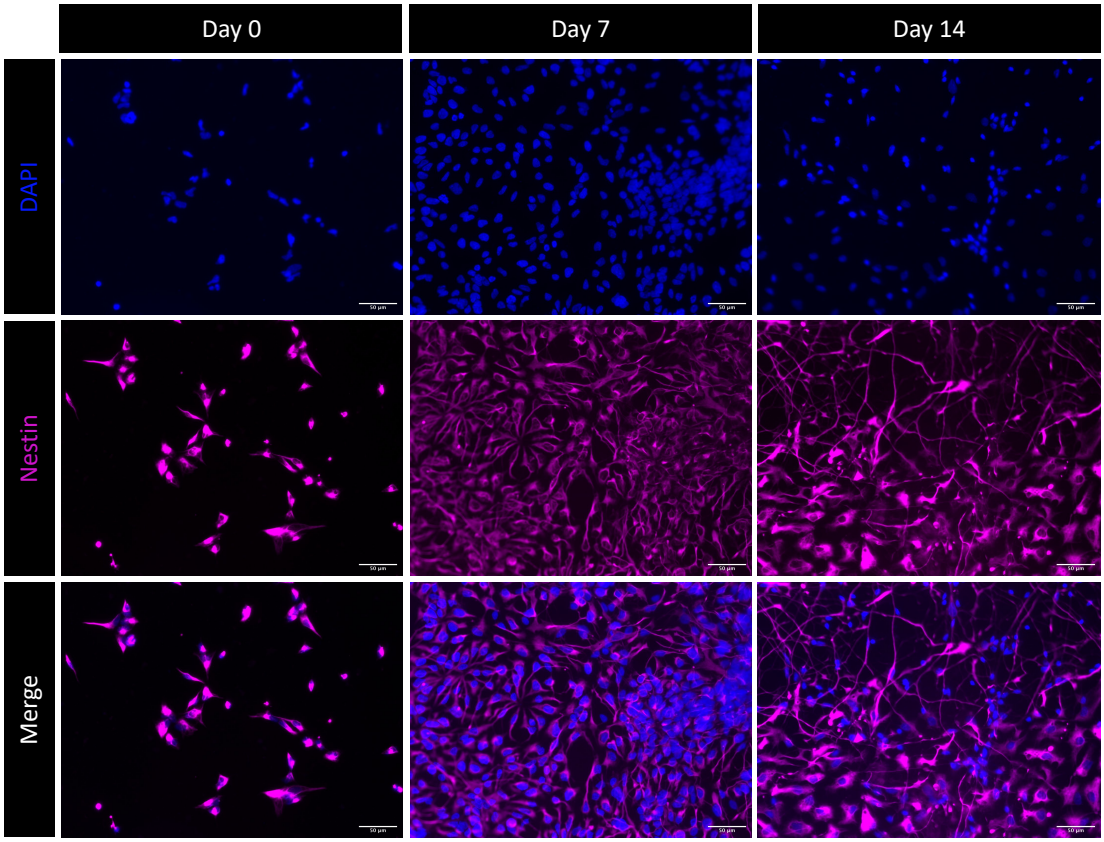

Figure 5-4. Transitional morphology of NPCs
developing into cortical neurons *(scale bar: 50μm).*

During the experimental window from day 14 to 21, the culture contained a mixture of NPCs (Nestin+) and cortical neurons (ß-III tubulin+) with minimal glial progenitors (GFAP+), representative images are shown in Figure 5-5.

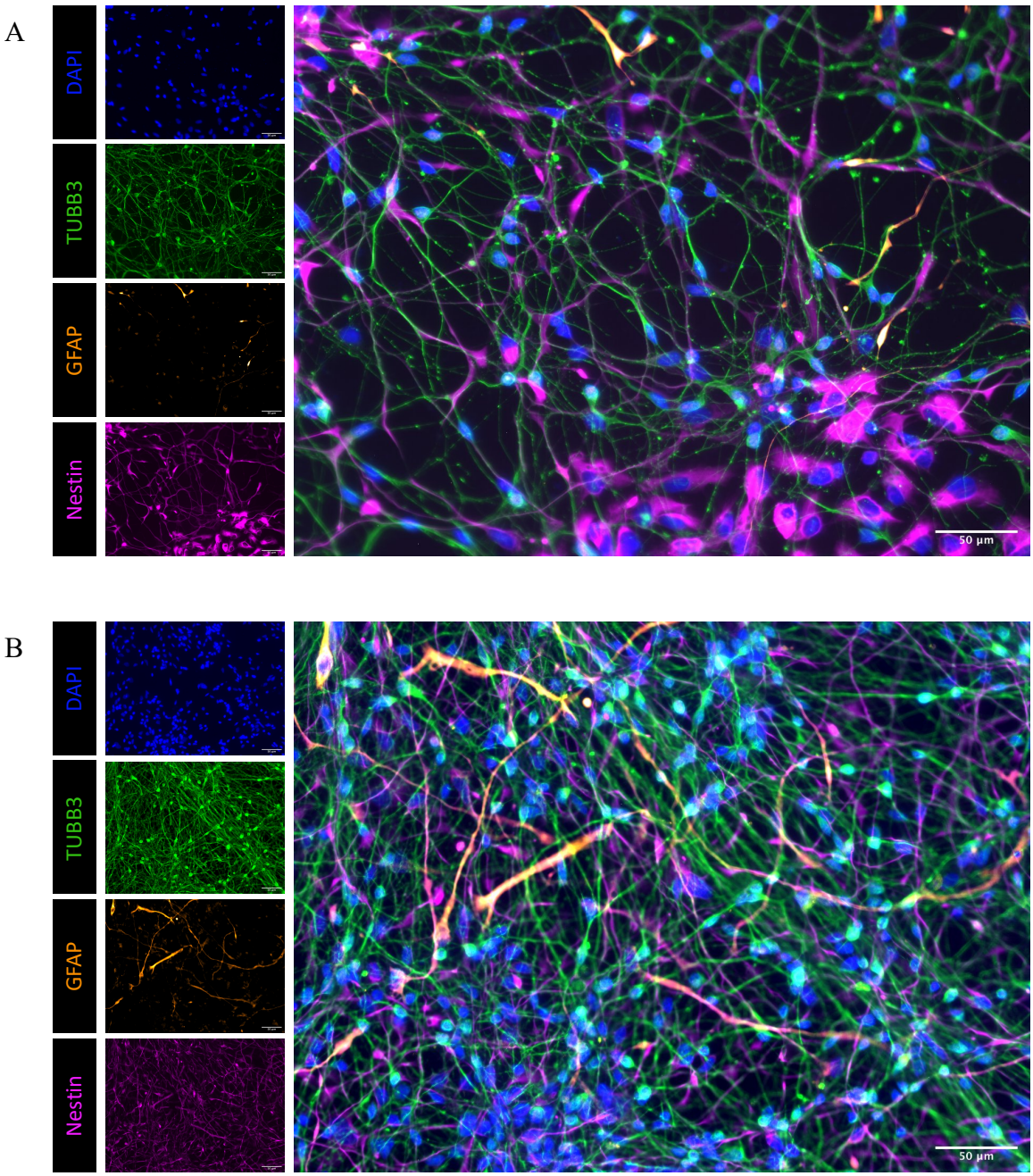

Figure 5-5. Representative images of cortical neuron cultures on (A) day 14 and (B) day 21 (scale bar: 50µm).

Quantitative immunohistochemistry performed on day 21 (n=5) confirmed a mixed population of NPCs (Nestin+ 51.28 +/- 5.16) and neurons (TUBB3+ 19.9 +/- 3.20) with minimal astrocytes (GFAP+ 4.9 +/- 1.10), shown in Figure 5-6.

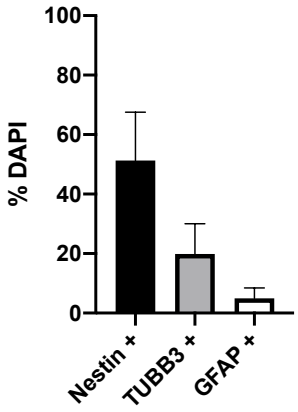

Figure 5-6. Quantification of cell culture population from immunohistochemistry on day 21 showing proportion of NPCs (Nestin+), cortical neurons (TUBB3+) and astrocytes (GFAP+). Data are represented as mean +/- s.e.m.

5.3.2 The effect of inflammation on cortical neurogenesis

To study cortical maturation in the context of inflammation, developing human iPSC-derived cortical neurons were exposed to cytokines associated with the neonatal systemic inflammatory response: C5a, IL-6, IL-8, TNF-α. Cultures were exposed for 7 days before immunostaining for markers of nuclear DNA (DAPI+), NPCs (Nestin+), cortical neurons (TUBB3+) and astrocytes (GFAP+).

5.3.2.1 Effect of cytokines on nuclei number

There were no significant differences in the mean nuclei number following exposure to cytokines when compared to control (Figure 5-7).

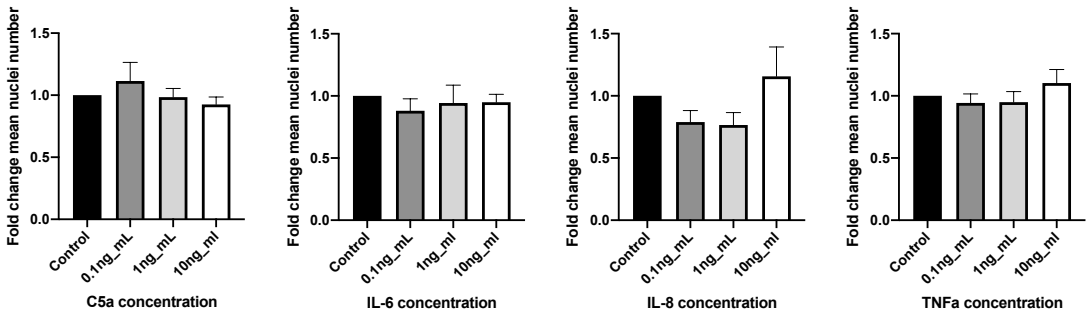

Figure 5-7. Quantification of mean nuclei number following exposure to C5a, IL-6, IL-8 and TNF-α.
Data are presented as fold change compared to control.

5.3.2.2 *Effect of C5a on cortical neuron differentiation*

C5a was associated with an increase in the proportion of TUBB3+ neurons following a 7-day exposure at a dose of 10ng/ml. Mean difference was -0.491 (95% CI -0.977 to -0.005), p=0.0470 (Figure 5-8). There were no significant differences in the population of Nestin+ NPCs or GFAP+ astrocytes. There was a trend towards a reduction in the proportion of mitotic cells using EdU labelling but this was not statistically significant (Figure 5-9). This suggests that the resulting increase in the proportion of post-mitotic neurons observed with exposure to C5a may be secondary to terminal differentiation rather than amplification of progenitors.

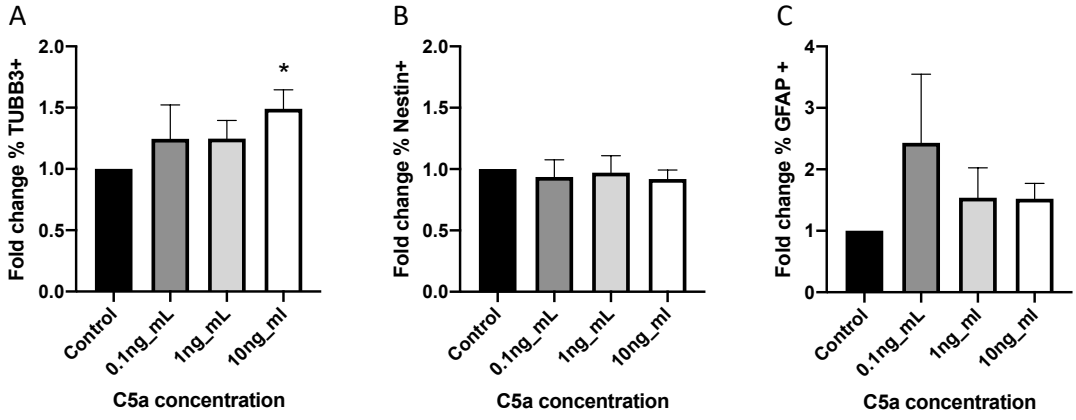

Figure 5-8. Quantification of cell population following exposure to C5a
(A) Proportion of TUBB3+ neurons, (B) Proportion of Nestin+ NPCs and (C) Proportion of GFAP+ astrocytes. Data are presented as fold change compared to control.

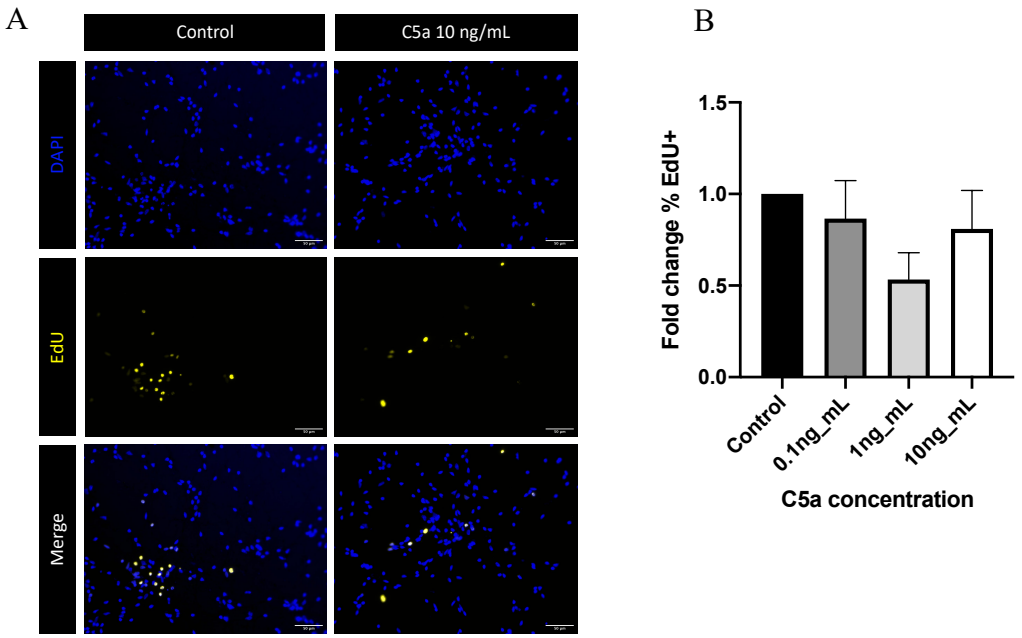

Figure 5-9. Quantification of mitotic cells using EdU labelling
(A) Representative images for 10ng/ml C5a vs control (scale bar: 50μm), (B) EdU quantification demonstrating fold change compared to control.

5.3.2.3 *Effect of IL-8 on cortical neuron differentiation*

IL-8 was associated with an increased proportion of Nestin+ NPCs following a 7-day exposure at a dose of 100ng/ml. Mean difference was -0.3259 (95% CI -0.561 to -0.091), p= 0.0032 (Figure 5-10). There was a trend towards an increased proportion of GFAP+ astrocytes but this was not statistically significant.

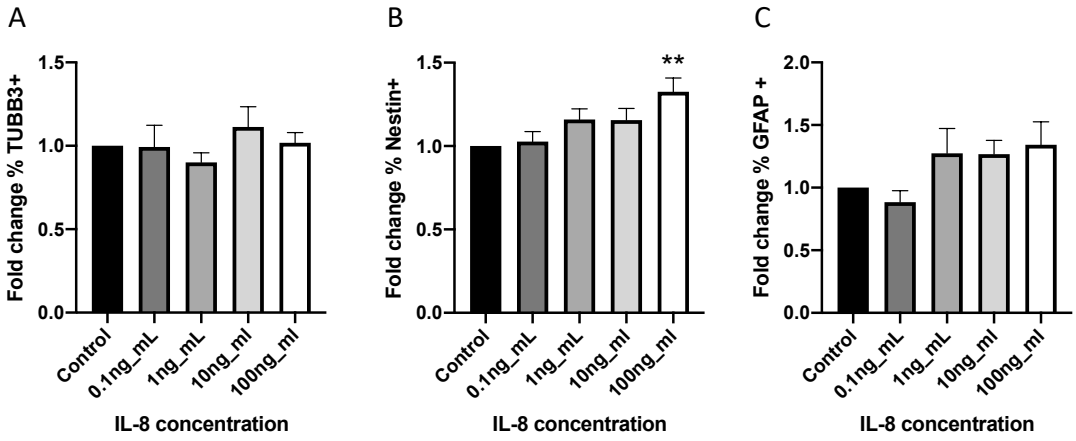

Figure 5-10. Quantification of cell population following exposure to IL-8
(A) Proportion of TUBB3+ neurons, (B) Proportion of Nestin+ NPCs and (C) Proportion of GFAP+ astrocytes. Data are presented as fold change compared to control.

5.3.2.4 *Effect of IL-6 on cortical neuron differentiation*

IL-6 also appeared to influence the proportion of GFAP+ astrocytes in culture but results were not statistically significant (Figure 5-11).

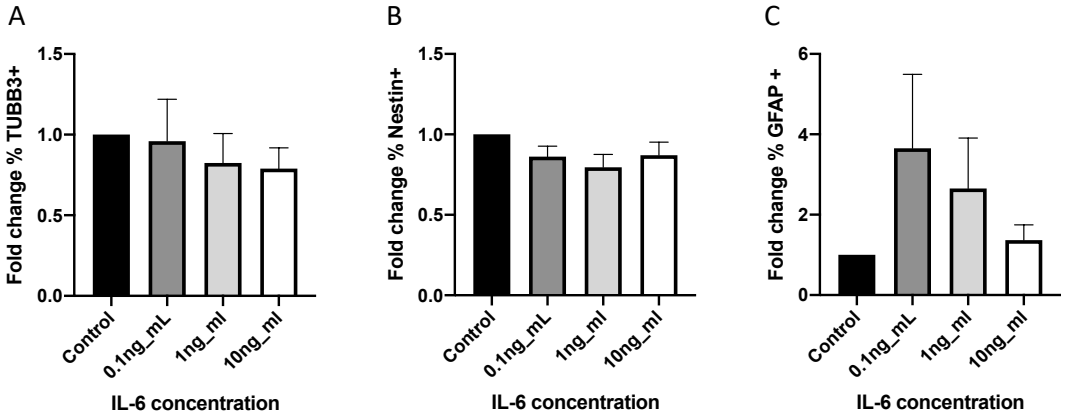

Figure 5-11. Quantification of cell population following exposure to IL-6
(A) Proportion of TUBB3+ neurons, (B) Proportion of Nestin+ NPCs and (C) Proportion of GFAP+ astrocytes. Data are presented as fold change compared to control.

5.3.2.5 *Effect of TNF-α on cortical neuron differentiation*

There were no significant differences in cell populations following exposure to TNF-α (Figure 5-12).

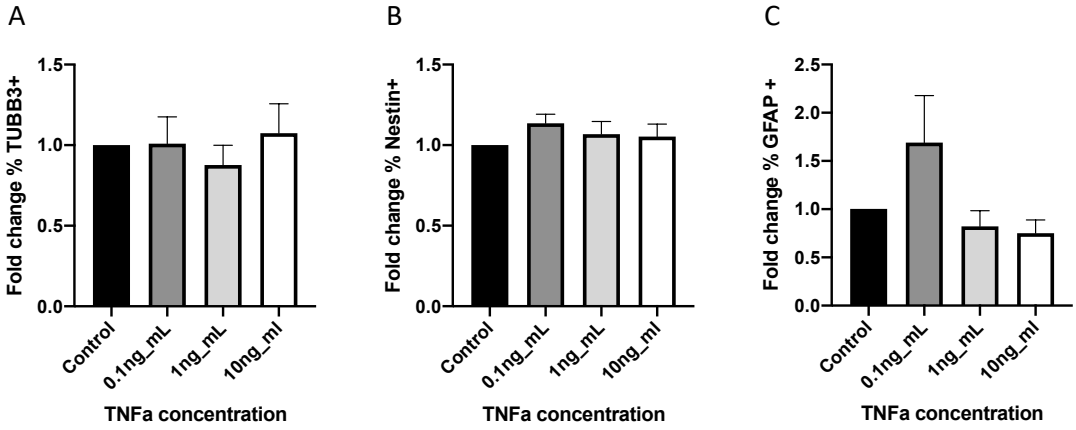

Figure 5-12. Quantification of cell population following exposure to TNF-α
(A) Proportion of TUBB3+ neurons, (B) Proportion of Nestin+ NPCs and (C) Proportion of GFAP+ astrocytes. Data are presented as fold change compared to control.

5.3.3 The effect of inflammation on neurite outgrowth

Due to cell density, the incorporation of GFP prior to platedown of NPCs enabled sparse labelling to facilitate quantification of neurite length. Figure 5-13 shows a representative image on day 21 of culture.

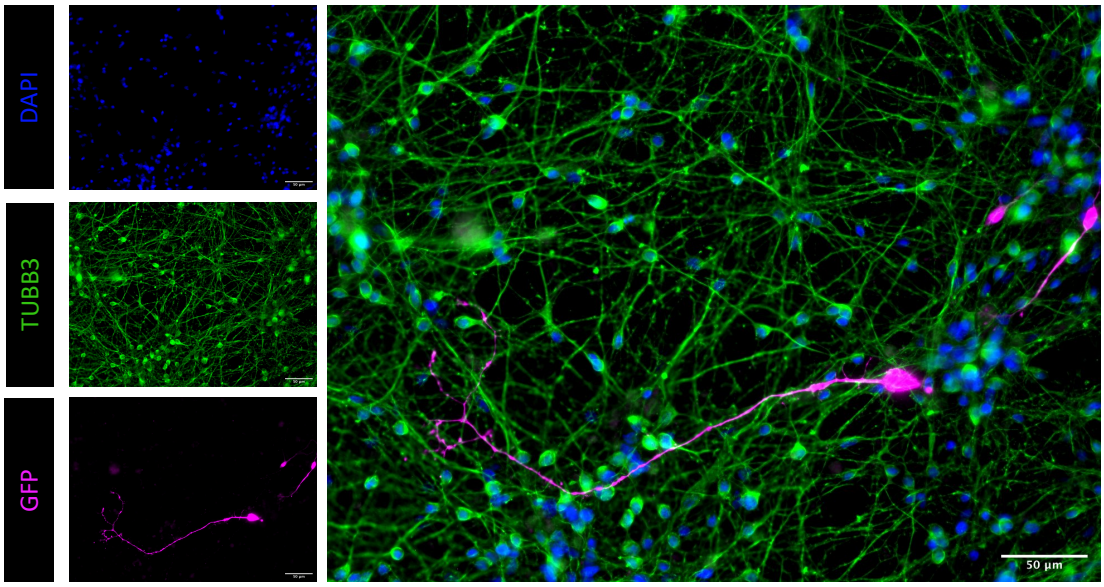

Figure 5-13. Representative image of GFP-labelled cortical neuron.

The mean number of GFP-labelled neurons examined per condition was 129 (range 81-172). The mean maximum neurite outgrowth lengths of cortical neurons exposed to 10ng/ml or 100ng/ml of C5a, IL-6, IL-8 and TNF-α are shown in Table 5-1.

		Mean maximum neurite outgrowth length, µm (SD)
Control		279.0 (116.0)
C5a	10ng/ml	259.7 (115.8)
	100ng/ml	182.9 (63.3)
IL-6	10ng/ml	284.9 (84.0)
	100ng/ml	239.1 (68.9)
IL-8	10ng/ml	177.2 (91.3)
	100ng/ml	210.6 (39.2)
TNF-α	10ng/ml	229.6 (71.22)
	100ng/ml	303.0 (115.2)

Table 5-1. Mean maximum neurite outgrowth lengths.

IL-8 was the only cytokine associated with reduced neurite length (Figure 5-14). Mean difference between control and 10ng/ml was 0.357 (95% CI 0.063-0.652), p=0.0176.

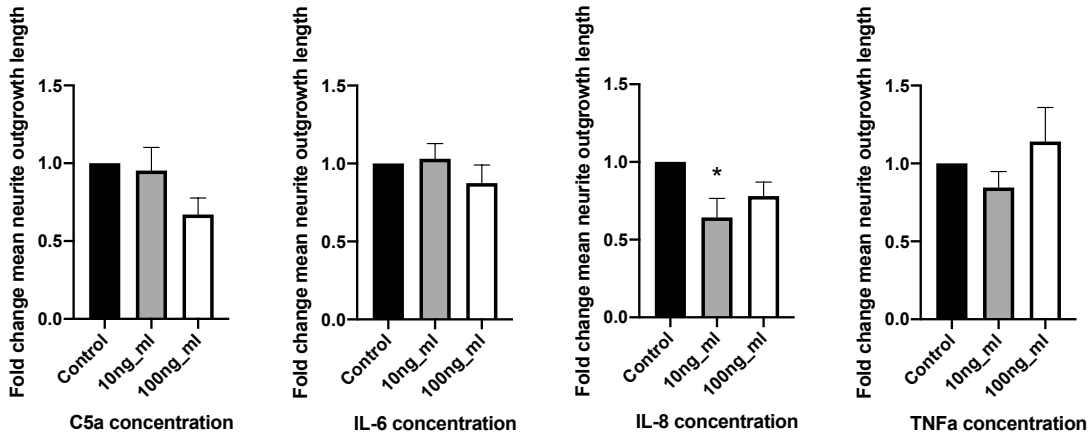

Figure 5-14. Fold change in mean neurite outgrowth length on exposure to 10ng/mL or 100ng/mL of C5a, IL-6, IL-8 and TNF-α. Data are presented as mean +/-s.e.m.

5.4 Discussion

In an iPSC-derived cortical neuronal culture system, exposure to C5a was associated with increased neurogenesis, whilst exposure to IL-8 was associated with NPC proliferation and reduced neurite outgrowth. These findings were not observed with exposure to other pro-inflammatory cytokines suggesting that C5a and IL-8 may exert cell-specific effects to alter cortical neurogenesis.

Complement C5a is a potent inflammatory mediator and a critical component of the innate immune response (Guo and Ward 2005) but accumulating evidence suggests that complement proteins are also involved in key biological processes of brain development and disorders of neurodevelopmental origin (Magdalon et al. 2020; Coulthard, Hawksworth, and Woodruff 2018). C5a acts via binding the G-protein coupled receptor C5aR, expressed by NPCs (Rahpeymai et al. 2006), cortical neurons (O'Barr

et al. 2001) and iPSCs (Hawksworth et al. 2014). In a mouse model of inflammation-induced preterm birth, C5a mediated glutamate excitotoxicity was found to contribute to cortical neuronal injury with C5aR blockade resulting in neuroprotection (Pedroni et al. 2014). Gene expression levels of C5a and the complement regulator SERPING 1 correlate with cortical thickness in humans (Allswede et al. 2018) and mouse embryos deficient in SERPING1 have reduced proliferation of intermediate progenitors, suggesting a role for the complement cascade in expansion of the neural progenitor pool in early cortical development (Gorelik et al. 2017). However, other studies have found that C5aR activation increased NPC proliferation through PKCζ/ERK signalling (Coulthard et al. 2017).

IL-8 is a key mediator of the innate immune response and acts via chemokine receptors, CXCR1 and CXCR2. Both receptors bind IL-8 with similar affinity to activate growth and stress kinases such as ERK1/2, JNK1 and p38, and induce intracellular calcium mobilisation from the endoplasmic reticulum to inhibit adenylyl cyclase and reduce AMP production. IL-8 has been shown to cause CXCR1-mediated death of NPCs (Kelland et al. 2011), whilst CXCR2 has been implicated in the maintenance of pluripotency and proliferation of human stem cells (Jung et al. 2015; Jiang et al. 2017). Pro-inflammatory cytokines and chemokines in sufficient doses have previously been shown to have negative effects on neurite outgrowth (Matelski et al. 2020). However, the mechanisms underlying this are not well understood. Neurite growth and guidance is regulated by multiple signalling pathways (Prem, Millonig, and DiCicco-Bloom 2020) involving extracellular factors (O'Kusky and Ye 2012), adhesion molecules (Jossin 2020; Miyamoto, Sakane, and Hashimoto 2015) and regulators of the cytoskeleton (Gallo 2013) which may be influenced by exposure to inflammation.

Wnt/ß-catenin signalling plays a key role in brain health and disease (Noelanders and Vleminckx 2017; Ng et al. 2019; Hoseth et al. 2018; Zhang, Sun, et al. 2012), including the development of white matter injury associated

with preterm birth (Van Steenwinckel et al. 2019). Canonical Wnt signalling is associated with activation of inflammatory downstream pathways (Moparthi and Koch 2019), including IL-8 in the context of cancer (Wen et al. 2020) and neuroinflammation (Robinson, Narasipura, and Al-Harthi 2019). Wnt activation leads to the expansion of neural precursors whilst inhibition has been associated with premature cell cycle exit and a reduction in the precursor pool (Chenn and Walsh 2002; Woodhead et al. 2006; Zhou et al. 2006; Srikanth et al. 2015).

Another pathway to consider is the interaction between mitochondrial metabolism and innate immunity, which has been recognised in the context of neuroinflammation and psychiatric disease (Culmsee et al. 2019). Exposure to pro-inflammatory proteins may influence mitochondrial functions which are fundamental to neurogenesis. The metabolic switch from glycolytic metabolism to oxidative phosphorylation appears to be an important regulator of stem cell commitment (Khacho and Slack 2018) and altered mitochondrial dynamics have been associated with defects in cell cycle exit, impaired neurite outgrowth (Nguyen et al. 2018) and cortical maldevelopment (Khacho et al. 2017).

Strengths of this study include: (1) the selection of biologically relevant concentrations of inflammation-associated proteins known to be differentially expressed in preterm infants when compared to term-born controls (Boardman et al. 2018) and elevated in the context of histologic chorioamnionitis (Hecht et al. 2011), FIRS (Costa and Castelo 2016) and neuroinflammation (Lee, Nagai, and Kim 2002), (2) the use of an established human culture system of an enriched neuronal population which is biologically relevant for the study of fetal and early postnatal cortical development, and (3) the principled selection of outcome measures based on established observations of reduced volume and altered cortical microstructure in preterm infants at term-equivalent age and the proposed influence of environmental factors on neural progenitor fate.

A limitation of the study is that the culture system did not allow the analysis of interneurons, which comprise 20% of the human cerebral cortex (Wonders and Anderson 2006). Inflammation has been associated with disrupted interneuron maturation in animal models and human preterm infants (Stolp et al. 2019) and culture systems to integrate both excitatory and inhibitory neuron populations may be important when modelling cortical injury, as Nitric oxide synthase expressing interneurons play a major role in neuronal death in response to cytotoxicity or ischaemia (Xu et al. 2016).

Whilst there were no significant changes in mean nuclei number observed under experimental conditions, no assessment of cell health/death assays were performed. Cell death is a recognised feature of brain injury associated with inflammation and molecular mechanisms should be considered in future study designs (Galluzzi et al. 2018; Truttmann, Ginet, and Puyal 2020).

Another limitation is that we could not investigate sex-specific effects as iPSCs were generated from a healthy female control. Environmental effects on brain development and immune function after preterm birth may be sex-specific (O'Driscoll et al. 2018; O'Driscoll, Greene, and Molloy 2017; Hanamsagar et al. 2017; Makinson et al. 2017), and long-term impact on brain growth has been described (Reiss et al. 2004; Kesler et al. 2008).

Enriched monolayer systems can provide valuable information about cell-specific effects but there are limitations to the use of 2D cultures which do not recapitulate the complexity and organisation of human brain development. The cell density is invariably lower than *in vivo* and the architecture and microenvironment are different. Given the fundamental role of glia in amplification of the neuroinflammatory response, organoid culture systems incorporating both astrocytes and microglia offer the most comprehensive model of early human brain development. Future work investigating the impact of environmental exposures on 3D systems will be key to the

interrogation of cellular targets linking systemic inflammation with altered cortical development.

5.5 Conclusions

These data demonstrate that clinically relevant doses of complement protein C5a and IL-8 are capable of influencing cell type diversity and neurite outgrowth in the developing neocortex. Whether these observations have functional consequences *in vivo* is yet to be determined and further work to identify the molecular and cellular mechanisms regulating proliferation and differentiation of radial glia and intermediate progenitors would provide important insights (Beattie and Hippenmeyer 2017).

As the sequential nature of progenitor diversification is critical to ensure optimal layering, organisation and efficient network formation within the cerebral cortex, these findings suggest that inflammation is implicated in the aetiology of cortical dysmaturation associated with preterm birth, focussing particular attention on the role of IL-8 which may have a specific impact on neurite growth.

6 Discussion

6.1 Summary of findings

Despite considerable improvements in survival rates after preterm birth, infants remain at high risk of brain injury and a range of adverse lifecourse outcomes. Whilst the role of inflammation in the encephalopathy of prematurity is well described, there are no therapeutic strategies available to reduce the risk of impairment.

The primary aim of this thesis was to identify specific immune mediators associated with atypical brain development after preterm birth to elucidate new targets for neuroprotective therapies. The integrated analysis of placenta, blood and brain MRI with human stem cell technology generated several key findings:

- Preterm infants have a distinct pro-inflammatory profile in umbilical cord blood when compared to term-born controls.
- Exposure to histologic chorioamnionitis was associated with alterations in the expression of several innate immune mediators in the umbilical cord blood of preterm infants but IL-8 proved to be the best predictor.
- Preterm infants who were exposed to histologic chorioamnionitis had an altered blood immune profile on postnatal day 5, suggesting that the systemic inflammatory response to intrauterine inflammation is sustained beyond delivery.
- Day 5 IL-8 concentration was associated with white matter microstructure, characterised by PSNDI, in preterm infants at term-equivalent age.
- In an iPSC-derived platform of cortical neurogenesis, complement protein C5a was associated with neuronal maturation, whilst IL-8 was associated with increased progenitor proliferation and altered neurite outgrowth.

As the histopathological diagnosis of chorioamnionitis is often delayed beyond the time of delivery, IL-8 offers a promising blood-based biomarker to identify exposure to intrauterine inflammation and activation of the neonatal systemic inflammatory response. Several studies have also shown that IL-8 is predictive of neonatal sepsis (Leviton et al. 2012; Zhou et al. 2015; Wong et al. 2008) and necrotising enterocolitis (Maheshwari et al. 2014; Benkoe et al. 2014). A chip-based immunoaffinity capillary electrophoresis (ICE) system has now been developed for measuring inflammatory mediators in dried blood spots with comparable accuracy to conventional immunoassays and could be utilised to quantify IL-8 at the cotside using small volume samples (Guzman and Guzman 2016; Phillips 2018). The coupling of highly selective antibody-capture with the resolution of capillary electrophoresis offers rapid, sensitive and cost-effective point of care testing with the potential to inform clinical decision making in the neonatal unit.
Given that FIRS is associated with increased risks of subsequent lung disease, bloodstream infection, and brain injury, it may be that infants who present with systemic inflammation and elevated IL-8 on admission to the neonatal unit require an individualised approach to reduce the risk of further inflammatory complications.

Previous studies investigating the relationship between immune mediators and brain imaging outcomes were limited to the identification of acquired brain lesions using cranial ultrasound and structural MRI. Through the use of diffusion MRI to characterise white matter development after preterm birth, Chapter 4 indicates that IL-8 may link systemic inflammation and white matter dysmaturation in preterm infants. IL-8 concentration on postnatal day 5 was associated with white matter microstructure characterised by PSNDI at term-equivalent age. This association may describe a relationship between the severity of systemic inflammation and white matter dysmaturation or highlight a specific role of IL-8. There are multiple potential mechanisms through which IL-8 dysregulation may disrupt healthy brain development but mechanistic studies to assess causation are warranted. This is particularly

important given the intricacy of immune responses. Individual mediators rarely function in isolation, instead contributing multiple roles within a complex system of cytokine networks.

The cellular composition and cytoarchitecture of the developing cerebral cortex are dependent upon proliferative expansion of the progenitor pool followed by a carefully timed switch to neurogenesis through a diverse array of progenitor subtypes including radial glia and intermediate progenitors. Mathematical modelling of cortical development suggests that altered progenitor output potential at different stages of fate determination could have major impact on cortical size and structural connectivity (Picco et al. 2018; Postel et al. 2019). Using a human iPSC-derived system to study cortical development, C5a was shown to influence the generation of excitatory glutamatergic neurons, whilst IL-8 was associated with increased progenitor proliferation and altered neurite outgrowth, suggesting that immune mediators associated with the systemic inflammatory response may exert cell-specific effects in the developing neocortex.
The discovery of altered neurite length associated with exposure to IL-8 may represent poor neuronal health, dysmaturation or a structural deficit. Given the importance of neurite outgrowth for dendritic arborisation, synaptogenesis and axonal guidance, future studies to identify the potential mechanisms and functional significance of this finding are warranted.

6.2 Strengths and limitations

This is the first study to integrate information about systemic inflammation from placenta and blood with biomarkers of white matter microstructure in preterm infants. The preterm birth cohort studied was representative of a typical preterm population in terms of antenatal factors and neonatal morbidity. An unbiased data driven approach to biomarker discovery was used and an imaging protocol using multi-shell acquisitions with high b-values enabled implementation of advanced biophysical profiling.

Peak width skeletonised water diffusion parameters were used as biomarkers of generalised white matter connectivity in view of their utility in discriminating preterm infants from term-born controls at term-equivalent age (Blesa et al. 2020). This thesis also demonstrates the utility of these metrics to investigate the impact of perinatal exposures on neonatal brain tissue development and their use in other neonatal populations at risk of altered brain development should be evaluated.

Histogram-based analyses of DTI and NODDI are computationally inexpensive and can be applied to combine data acquired from different scanners facilitating their potential application as biomarkers in future clinical trials of neuroprotection.

Whilst PSMD has been associated with processing speed and cognitive ability in adults (Deary et al. 2019; Wei et al. 2019), it will be important to assess long-term outcomes in order to characterise the neurodevelopmental correlates and predictive performance of PSMD and PSNDI in children who were born preterm.

A limitation of the work presented in this thesis is that maternal immune influences and risk or resilience conferred by the genome or epigenome were not able to be examined. Studies investigating the maternal immune activation hypothesis have shown that maternal inflammation is associated with offspring brain development (Rudolph et al. 2018; Wu et al. 2017; Al-Haddad et al. 2019; Estes and McAllister 2016). Placental signalling also plays a role in neurodevelopmental programming through altered gene expression and epigenetic responses to fetal and maternal environmental signals (Shallie and Naicker 2019).

Prematurity may represent an environmental influence which exposes underlying genetic susceptibility to brain injury resulting from neuroinflammation. Genome-wide association studies have identified variants associated with prematurity (Zhang et al. 2017) and genetic susceptibility to brain injury after preterm birth has been described (Boardman et al. 2014; Krishnan et al. 2016; Krishnan, Van Steenwinckel, et al. 2017; Krishnan,

Wang, et al. 2017). Dynamic epigenetic mechanisms including DNA methylation, histone modifications, chromatin remodelling and non-coding RNAs are involved in the regulation of gene expression during neurodevelopment (Spiers et al. 2015) and the co-ordination of innate immune responses (Zhang and Cao 2019). These processes can be influenced by early exposure to the extrauterine environment and altered in the context of perinatal brain injury (Sparrow et al. 2016; Provenzi, Guida, and Montirosso 2018; Everson et al. 2019; O'Sullivan et al. 2019).

Another limitation is that sample size did not permit sub-group analyses based on gestational age or sex. There is an increased risk of fetal inflammation with lower gestational age and many of the complications of prematurity are encountered more frequently in infants at the threshold of viability. This may be explained by immune dysregulation or may simply reflect increased vulnerability of developing organ systems. Environmental effects on brain development and immune function may be sex-specific, particularly given the role of microglia as both targets and drivers of brain sexual differentiation (O'Driscoll et al. 2018; O'Driscoll, Greene, and Molloy 2017; Hanamsagar et al. 2017; Makinson et al. 2017).

6.3 Future directions

The development of therapeutic strategies targeting inflammation in preterm brain injury has been hindered by difficulties in accessing primary brain tissue and a failure of animal models to fully recapitulate human neurobiology. 3D organoids offer an exciting tool to study human brain development, particularly dynamic functions such as neuronal migration and cortical layering (Lancaster et al. 2013). Organoids with a mixed culture of neuroectoderm, microglia and endothelial vascular cells offer a more representative model of early human brain development with the added ability to interrogate the role of crosstalk between neurons and glia in the context of immune activation (Marton and Pașca 2020; Krencik et al. 2017;

Muffat, Li, and Jaenisch 2016; Madhavan et al. 2018). Bioengineering techniques may also provide additional opportunities to enhance organoid culture systems using microfluidic channels to control the microenvironment (Lovett et al. 2020) .
3D cortical organoids follow the pace of human fetal brain development (Paşca et al. 2015) and have been utilised to study neonatal brain injury in the context of hypoxia-ischaemia (Boisvert et al. 2019; Pașca et al. 2019). An organoid platform relevant to early fetal neurodevelopment will enable the study of both environmental and genetic factors underlying preterm brain injury with reproducibility and scalability, both of which are key to developing high throughput drug screening models (Yoon et al. 2019; Velasco et al. 2019).

This thesis focusses attention on the role of complement and IL-8 in linking systemic inflammation with preterm brain dysmaturation. Given the complex role of the immune system in key processes of neurodevelopment, further understanding of the underlying molecular and cellular mechanisms alongside studies of the functional consequences of the phenotypes observed are key next steps to exploring their potential as targets for immunomodulation. It will also be important to validate these observations with a replication cohort, ideally complemented by transcriptional profiling. Previous pathway analyses using whole blood RNA showed a distinct immune metabolic signature of neonatal infection (Smith, Dickinson, et al. 2014) but fetal inflammatory responses have not yet been fully characterised.

Given the role of inflammation in white matter dysmaturation, another exciting area of future development is the refinement of myelin imaging techniques for neonatal application. The Theirworld Edinburgh Birth Cohort imaging protocol was recently extended to include a magnetisation transfer ratio (MTR) with successful acquisition of images from preterm infants and term-born controls. Using the ratio between free water and water with restricted motion due to macromolecules we will be able to describe myelin deposition in the

developing brain with potential for future application in studying the impact of perinatal exposures such as inflammation and nutrition.

The emerging field of immunoneuropsychiatry recognises the importance of early life inflammatory exposures on immune programming and brain health across the lifecourse (Pape et al. 2019; Al-Haddad et al. 2019; Rychlik and Sillé 2019) and the long-term follow up of babies in this cohort will facilitate knowledge discovery in this area. Thorough the combination of standardised developmental assessments, linkage to routinely collected datasets and longitudinal MRI brain scans in childhood and adolescence, there will be an opportunity to characterise early neural and immune signatures of later psychiatric disease associated with preterm birth.

The quest to find reliable imaging and blood-based biomarkers predictive of long-term outcomes remains a challenge within neonatal medicine, but it is likely that a multiparametric approach will be required to enable early identification of infants at risk who could benefit from targeted intervention. Combining organoid technology with the power of -omics approaches may facilitate the discovery of specific factors associated with risk or resilience to inflammation and brain injury. In the future, iPSCs could be produced from somatic cells of participants and used to generate organoids to interrogate neuropathology, elucidate cellular targets and test potential therapies, offering the possibility of truly individualised precision medicine.

Cohort retention is crucial to the realisation of these objectives and creative solutions to maintaining participant engagement during a global pandemic will be required.

6.4 Conclusions

This thesis contributes three studies which provide novel insights into the role of inflammation in the aetiology of cortical dysmaturation and altered white matter microstructure associated with preterm birth. There appears to be a window of opportunity for therapeutic intervention using anti-inflammatory or immunomodulatory therapies after preterm birth and future work should focus attention on IL-8 as a potential therapeutic target.

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